STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

VI. Biology of Hemolytic Streptococcus: Antigenic Relationships between Strains of the Scarlatinal and Erysipelas Groups.

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In a recent study (1) of agglutination and absorption of agglutinin with strains of hemolytic streptococcus from scarlet fever and erysipelas, we found these two groups to be related antigenically. This relationship was peculiar in that the strains within the groups were not identical, yet possessed of common antigenic properties. These common antigenic properties were apparently not entirely group-specific, since sera of one group would agglutinate certain strains of the other group of streptococci. When several erysipelas strains and their corresponding sera were employed in a comprehensive series of agglutination reactions, in which each of the strains was tested for agglutination with each of the sera, few negative reactions were found. Except with strains which were apparently physically incapable of agglutination, the reactions within the group were consistently positive with each strain and serum. When scarlatinal strains were agglutinated with these erysipelas immune sera, by testing each of the scarlatinal strains for agglutination with each of the sera in a manner similar to the tests carried out with the erysipelas strains, a much lower percentage of positive reactions was obtained. None of the strains and none of the sera showed consistently positive reactions. A strain might agglutinate in one or several of the erysipelas sera. A serum might agglutinate one or more of the scarlatinal strains. The antigens of the two groups were apparently related.

Absorption of agglutinin has shown that the strains within the groups are not identical, since it is not usually possible to absorb the agglutinin for the homologous strain from serum with heterologous

group strains. However, when a serum is absorbed with an heterologous strain of a group, and the absorbing dose is equivalent to the dose of the homologous strain necessary for the absorption of the homologous agglutinin, all heterologous agglutinin is removed. Such absorption not only removes the agglutinin for heterologous strains of the group represented by the serum, but the agglutinin for strains of the other group as well. It is only by excessive and repeated doses of the absorbing antigen under conditions where the agglutinin is liable to deteriorate from long continued heating that the homologous agglutinin can be absorbed by heterologous group strains. Apparently the antigenic characteristic of the individual strain is paramount while the group characteristics are of secondary importance in the bacterial structure.

The most satisfactory explanation for the antigenic relationships apparently existing between the strains of the erysipelas and scarlatinal groups, is that the antigen in each strain is a mosaic of antigenic fractions which occur in variable amounts in different strains. Group specificity would then depend on the presence of characteristic fractions some of which might occur in certain strains of the other group. The predominating fraction would give the strain specificity, a characteristic antigen or group of antigens would give group specificity, and the occurrence of one or more of these fractions in both groups would explain the apparently existing relationship between scarlatinal and erysipelas strains.

We have previously studied the absorption of agglutinin by several methods. In 1924 we studied the scarlatinal group (2). We attempted to absorb the agglutinin for the homologous strain from its corresponding serum with heterologous scarlatinal strains. We were able to accomplish our purpose but with certain reservations as far as strains and sera were concerned. The following quotation is taken from the published article.

> "We have found that certain combinations of sera and strains are necessary for the successful absorption of agglutinin. These combinations can usually be picked out by the speed of the reaction and the degree of agglutination. Strains will absorb the agglutinin from sera which are fresh if the agglutination is complete within 15 minutes at 55°C.
>
> By selecting sera and strains which have reacted vigorously we have been able to obtain a series of absorption tests in which every strain is represented."

These reactions were carried out with doses of antigen which we now consider excessive for absorption work. In an immediately previous article published in 1926 (1) the dose of antigen employed in absorbing the sera was uniform. It was a dose slightly larger than that required when the homologous agglutinin was absorbed from a serum for the corresponding strain. With this dose it was seldom possible to absorb the homologous agglutinin with heterologous strains of the group. While these absorption tests demonstrated what all the strains of a group were not identical they did not indicate how closely the individual strains might resemble each other. A series of absorption reactions has been carried out in which the sera were absorbed with doses of antigen uniformly and gradually increased from the unabsorbed serum ("zero" dosage) to the dose necessary for the absorption of the homologous strain. Both scarlatinal and erysipelas strains have been included so as to determine the relationship between the strains in the erysipelas group as well as their relationship to those strains of the scarlatinal group which agglutinate in erysipelas immune sera.

Experimental Methods.

Three erysipelas immune sera and a number of erysipelas and scarlatinal strains were selected for the agglutination and absorption tests. The erysipelas strains were chosen without discrimination but scarlatinal strains were picked which agglutinated with the three sera. Since agglutination of each of these scarlatinal strains did not occur with all of the sera, the same strains could not be employed throughout the experiments. The technique of immunization and agglutination have been fully described previously (1, 2). The method of absorption also has been described with the exception of the technique for obtaining uniform absorptive doses and of the manner in which the fractional doses were estimated.

The physical bulk of streptococci necessary for the subminimal absorption of agglutinin was determined for each serum and the homologous strain. The streptococci were grown in broth and centrifuged under uniform conditions in especially constructed quantitative centrifuge tubes. The quantities necessary with each of the sera and the homologous strain were averaged, and this average was accepted as the unit absorptive dose of bacteria for a definite volume of serum of 1:20 dilution. Each of the three erysipelas sera was then absorbed with fractional and multiple doses of this unit. Each of the three sera was absorbed in turn by all three strains represented by the immune sera. After each absorption the sera were tested for residual agglutinin with erysipelas and scarlatinal strains.

The results of these absorptions and agglutinations are arranged in Tables I to III. Each table represents the work with a single serum. The absorbing strain is indicated at the head of each broad column and the decimal part of the unit absorbing dose employed is found at the left margin. The numerals at the intersecting points show the degree of agglutination with the absorbed serum and the various scarlatinal and erysipelas strains. The agglutinations were done in a series of six dilutions, 1:40, 1:80, 1:160, 1:320, 1:640,

TABLE I.

Fractional Agglutinin Absorption: Erysipelas Serum I Absorbed with Erysipelas Strains I, II, and III.

Absorbing dose	Absorbed by Strain E I								Absorbed by Strain E II								Absorbed by Strain E III							
	E I	E II	E III	E VII	S I	S VIII	S IX	S XV	E I	E II	E III	E VII	S I	S VIII	S IX	S XV	E I	E II	E III	E VII	S I	S VIII	S IX	S XV
1.5	—					—			5	—	—		—											
1.0*	3					1			5	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—
0.5	6	—	—	—	—	2	—	—	5	—	—	—	—	—	—	—	4	—	—	1	—	—	—	—
0.45																								
0.4	6	—	—	3	—	3	—	1	5	—	—	—	1	1		—	6	—	—	3	—	1	—	—
0.35																								
0.3	6	—	2	2	1	3	—	2	6		1	—	2	1		—								
0.25	6	—	1	3	1	3	—	3	6	—	1	1	3	4	—	1	5	—	—	4	3	3	—	4
0.2	6	2	2	3	—	4	—	2	6	—	4	3	3	4	—	1	5	—	1	6	4	4	1	4
0.15	6	2	2	5	4	5	1	5	6	—	5	3	3	4	—	3	5	1	1	6	5	5	3	5
0.1	6	2	4	6	4	4	3	4	6	1	6	5	5	4	1	4	6	1	2	6	5	5	4	6
0.05	6	4	4	6	6	6	2	5	6	2	6	6	6	6	3	4	5	1	2	6	5	6	4	6
0.0	6	5	6	6	6	6	3	6	6	4	6	5	6	6	3	6								

* Absorbing doses are in fractions and multiples of this unit.

and 1:1280. The numerals 1, 2, 3, 4, 5, and 6 indicate respectively the last dilution in which agglutination occurred. The scarlatinal and erysipelas strains have been indicated by the appropriate letters S and E.

DISCUSSION AND SUMMARY.

The interpretation of the results in these experiments is difficult on account of the variations in the physical capacity of different strains of hemolytic streptococcus to agglutinate and absorb agglu-

tinin. Even when the antigens for agglutination and absorption are standardized and sera are carefully titrated variations in agglutination occur with different strains and sera. Variations may occur because of differences in the titer of the immune sera employed. In a large series of reactions with the same sera and strains, errors in interpretation depending on these factors can be eliminated because strains which agglutinate poorly will consistently give weak reaction with all sera, and weak sera will agglutinate all strains poorly. After such varia-

TABLE II.

Fractional Agglutinin Absorption: Erysipelas Serum II Absorbed By Strains E I, II, and III.

Absorbing dose	Absorbed by Strain E I										Absorbed by Strain E II										Absorbed by Strain E III									
	E II	E III	E VI	E VII	E IX	S I	S VIII	S IX	S XI	S XV	E II	E III	E VI	E VII	E IX	S I	S IV	S VIII	S IX		E II	E III	E VI	E VII	E IX	S I	S VIII	S IX	S XI	S XV
1.5	4				—			—		—											5			—	—			—		
1.0*	6	—			4			—		—	1				—				—		6	—	—	—	—	—	—	—	—	—
0.5	6	—	—	—	4	—	—	—	—	—	4				—				1		6	—	—	—	2	—	—	—		—
0.45																					6	—	—	—	3			—		—
0.4	4	—	—	—	4	—	—	1	—	—	5	—	—	—	—						6				3			—		—
0.35				—	4	—	—	3	2	—	6	—			—				4					—	3			—		—
0.3	6	1	—	—	4	—	—	5	4	—	6	1			—				4					1	3			1		—
0.25	6	5	—	—	6	—	1	6	6	—	6	3	—	—	—	—	—	—	6		6	—	—	1	3	—	—	3	—	1
0.2	6	6	—	1	6	—	2	6	5	1	6	3	—	—	3	—	—	—	6		6	—	—	3	4	—	1	4	1	1
0.15	6	6	1	3	3	1	6	6	6	2	6	3	—	—	5	—	—	—	6		6	—	2	4	4	—	1	5	3	3
0.1	6	6	2	3	6	3	6	6	6	4	5	4	2	—	5	—	—	4	5		6	3	1	5	6	1	3	4	4	3
0.05	6	6	3	4	6	4	6	6	6	3	6	6	6	4	6	2	2	4	6		6	3	4	6	6	3	6	6	6	4
0.0	6	6	4	5	6	4	6	6	5	4	6	6	5	6	6	4	4	6	6		6	6	5	6	6	3	6	6	6	3

*Absorbing doses are in fractions and multiples of this unit.

tions in agglutination are eliminated, if a strain varies grossly in agglutination with different sera, or the titer of different sera varies with the same strain as in Tables I to III, variations which occur must represent antigenic dissimilarities.

We have previously commented on the dissimilarity of the individual strains in the scarlatinal and erysipelas groups. Although the strains within each of the groups are related they are seldom identical. This individuality of the strain was indicated by the fact that absorp-

tion by heterologous strains failed to absorb the agglutinin for the strain homologous with the serum. This strain individuality is again apparent in Tables I to III. Doses of heterologous strains equivalent to the unit absorptive dose which has already been defined, fail to remove the agglutinin for the homologous strain. This dose is sufficient however, to absorb the agglutinin for practically all heterologous strains. It appears from this observation that the strain specificity dominates the group specificity.

TABLE III.

Fractional Agglutinin Absorption: Erysipelas Serum III Absorbed with Erysipelas Strains I, II, and III.

Absorbing dose	Absorbed by Strain E I										Absorbed by Strain E II										Absorbed by Strain EIII									
	E I	E III	E VI	E VII	E IX	S I	S III	S VI	S VIII	S XV	E I	E III	E VI	E VII	E IX	S I	S III	S VI	S VIII	S XV	E I	E III	E VI	E XV	S I	S III	S VI	S VIII	S XV
1.5	—	3			—						—	2						—											
1.0*	—	2			—						—	2						1	—	—	—	—	—	—	—	—	—	—	—	—
0.5		3			—			—		—		2	—					1			—	—	—	—	—	—	—	—	—	—
0.45	—	3	—	—	—	—	—	—	—	—	—	4	1	—	—	1	—	1	1	—	—	1					1	—	—
0.4																														
0.35	—	3	—	—	1	—	—	2	—	—	1	2	3	—	—	4	—	3	2	—	1	4					1	—	—
0.3																														
0.25	1	4	3	—	3	1	—	2	—	—	3	3	1			2	1	1	4	2	1	3	—	—	—	—	—	2	1
0.2	1	5	3	—	3	—	—	4	1	2	4	5	1			2	2	3	4	2	1	4	3	1	1	—	5	3	1
0.15	4	5	4	—	5	3	1	3	4	1	5	4	2		1	4	1	5	3	4	4	5	3	1	1	1	4	4	3
0.1	5	4	4	1	6	3	2	5	5	3	5	6	2		4	5	4	4	5	4	4	5			3	3	4	4	4
0.05	5	5	6	3	6	4	4	5	6	5	4	5	4		4	6	4	5	6	6	5	6			4	3	5	4	4
0.0	6	6	6	4	6	5	3	6	6	5	6	6	4		5	6	5	5	5	6									

* Absorbing doses are in fractions and multiples of this unit.

Between the unit absorptive dose and a dose which is approximately 0.2 to 0.3 of this unit, is a zone of great variation in absorption and agglutination. Below this zone absorption is complete for few strains. The differences in titer of various strains and absorbed sera in the zone where variations occur are very definite. For example in Table II, Strain E IX agglutinates well in Serum II absorbed with Strains E I and E III but poorly in serum absorbed with Strain E II. Numerous similar examples may be found. In a few instances heterologous strains agglutinate nearly as well as the homologous strains in these

absorbed sera. Such strains probably resemble the homologous strains closely. These resemblances are not confined to the erysipelas strains but occur among the scarlet strains which agglutinate in erysipelas sera. Apparently it is impossible to distinguish such scarlatinal strains from erysipelas strains by absorption of agglutinin.

The relationship shown by these absorption tests is additional evidence in favor of the mosaic nature of the antigen. Strains of streptococcus of the erysipelas groups are apparently composed of several agglutinogenic fractions. If the antigen of a strain is made up of the fractions A, B, C, D, and E, serum for that strain would contain A, B, C, D, and E agglutinins. If this serum were absorbed with the homologous strain or an identical heterologous strain the agglutinin would be completely absorbed. On the other hand a qualitative or quantitative difference between the two strains would be apparent in the absorption and agglutination reactions. Such differences are evident in Tables I to III. This serum would agglutinate any strain containing any one or several of the fractions, but absorption of the serum by such a strain would leave fractions of the agglutinin in the serum. This absorbed serum would agglutinate the homologous strain. Heterologous strains would agglutinate if fractions remained in the serum corresponding to their antigenic components. Hence the reactions in the absorbed serum would depend on the qualitative and quantitative relationships between the component fractions in the serum, the absorbing strain, and the strains agglutinated.

This conception of multiple antigens is not new in bacteriology. Durham (3) explained the reactions in the colon-typhoid group of bacilli on this basis. More recently Durand and Sédallian (4), and Andrewes, Derick, and Swift (5) have expressed the opinion that the agglutination reactions with hemolytic streptococcus can only be accounted for in this way. In many respects the reactions observed with the colon-typhoid group of Gram-negative bacilli resemble those experienced with the erysipelas and scarlatinal groups of hemolytic streptococcus. Apparently we have exhausted the possibilities of studying these groups further by agglutination and absorption. Our knowledge regarding the specificity and relationship of the antigenic fractions must come from the study of fractions isolated and refined by chemical methods.

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EFFECTS OF LIGHT ON NORMAL RABBITS, WITH ESPECIAL REFERENCE TO THE ORGANIC REACTION.

I. Clinical and Postmortem Observations.

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For several years a study of the animal organism in these laboratories has included an investigation of the mass relationships of various organs with especial reference to the subjects of variation, the effect of environmental influences, and the relation of these conditions to phenomena of animal resistance as indicated by the occurrence and general course of disease.

A striking feature both of experimental syphilis and of a transplantable neoplasm of the rabbit is the variability of the disease manifestations, and observations over a period of several years have led us to the opinion that a relationship exists between the season of the year and the general character of the disease. It was further found that the weights of many organs not directly involved by the disease process were affected and that these alterations could be correlated with the character and clinical course of the disease. In addition, variations of a rhythmic character which appeared to have a seasonal significance could be demonstrated in the organ weights of normal rabbits, and the organs in which these changes were especially marked were those in which the more pronounced weight alterations were observed in association with syphilis and the malignant tumor.

The influence of the seasonal factor has been interpreted as affecting animal economy, while susceptibility or resistance to disease has been considered as a function of the animal organism. Among the several factors contributing to weather conditions during the time of our experiments, sunshine appeared to be most closely correlated with variations in disease manifestations. That is, the actual hours of sun-

shine together with the rate, extent, and persistence of change in the curve of sunshine over a given period could be related to the character of the experimental diseases. Furthermore, when the curve of sunshine was compared with the organ weights of a sufficiently large number of normal rabbits examined during the time of our experiment, it was found that the fluctuations of the curve corresponded with variations in the mass relationships of practically all the organs, and perhaps most strikingly so in the case of certain of the ductless glands and representative lymph nodes. Since our experiments were in each instance carried out with rabbits kept in rooms which received practically all sunlight only as filtered through ordinary window glass, the effects observed cannot be attributed wholly to the short or so called therapeutic ultra-violet rays.

The observations led to the conception of a relationship between the nature or character of light, on the one hand, and the physical state and functional activities of the animal organism, on the other. In order to test this conception experimentally, an investigation was undertaken in which conditions of light could be controlled. Since the significant features of the factor of sunlight appeared to be its total amount together with the change in actual hours of sunshine, our initial experiments were carried out with a constant illumination of the maximum intensity that was practicable with a simple equipment. Its effect was studied, first, upon the normal rabbit with especial reference to organ weights, and second, upon the course of the malignant tumor and of experimental syphilis. A summary of the first experiment dealing with the results of tumor inoculation has already appeared (1), and those in which *T. pallidum* was employed will be published shortly.

The results of the experiment with normal rabbits are reported in the present series of papers. The methods employed, the clinical observations, and the postmortem findings are described in Paper I. In Paper II, the actual organ weights and the weights of organs per kilo of net body weight are presented in tabular form. An analysis of these statistical data, including a consideration of their reliability as an index of existing conditions, is discussed in Paper III.

Methods and Materials.

The rooms in which this experiment was carried out were three in number. One, the so called "light" room, measuring 19 × 9.5 × 10.25 feet or including 1850 cubic feet, was kept constantly and solely illuminated by 1000 watt Mazda lamps and Cooper Hewitt 50 inch low pressure mercury arcs, Type P, in crown glass, arranged in three superimposed horizontal rows on the long axis of the room. Each row contained four Mazda lamps and one mercury arc. The animal cages were placed in racks along the sides of the room parallel with and equidistant from the source of lighting. The intensity of the light reaching the cages averaged 425 foot candles; these determinations were made by the Electrical Testing Laboratories to whom our appreciative thanks are due. The spectrogram supplied by the Cooper Hewitt Company shows that the crown glass of the mercury arc does not transmit light below wave-lengths of 3022–28 Ångström units, while a spectrogram of the Mazda lamps which was kindly made for us by Dr. Frederick L. Gates shows that their light is cut off at about 3100 Ångström units. For the purpose of the present experiment there was no need to analyze further the nature or quality of the light.

In the "dark" room all light was constantly excluded except for the time, approximately 1 hour per day, when the animals were fed or examined. On these occasions a single 30 watt Mazda lamp was used. Satisfactory ventilation and temperature regulations of both the light and dark rooms were obtained by a system of fans and ventilating vents.

The "control" room was an unaltered animal room; it has a southern exposure and is lighted by two large windows. During the time of the experiment practically all the sunlight in this room diffused through the ordinary lead glass of these windows.

The experiment was conducted as follows: 150 male rabbits were assembled from November 14 to December 15, 1924, separately caged, and kept in ordinary animal rooms. The rabbits were chosen from carefully selected stocks of well nourished animals free from any external manifestations of disease. They were obtained chiefly from breeders or dealers in eastern Pennsylvania and included all the ordinary breeds of rabbits that are commonly used in our laboratory. The various breeds were proportionately distributed in the major divisions and subgroups of the experiment. The exact age of the animals was unknown but the great majority of them were between 8 months and 1 year old. The diet of all was the same throughout the experiment and consisted of hay, oats, and fresh cabbage or carrots.

On December 31, 1924 and January 2, 1925, 50 rabbits were placed in the light room, 50 in the dark room, while the third lot of 50 animals remained in the control or unaltered animal room. Groups of 10 rabbits from each room were killed and examined 2, 4, 6, 8, and 12 weeks later.[1] In addition, other groups of normal

[1] Data for a 10 week period were obtained from additional groups of rabbits placed under the same conditions on January 29 and killed on April 8,

rabbits designated as "outside" controls were killed and examined at the same time.[2] These animals had been in the laboratory for only a few days and represent a stock which had been living under unknown but presumably different conditions from those of the control rabbits in the experiment.

Upon the day the rabbits were killed the feeding was omitted; they were weighed and then killed by air embolism, a method quicker and less disturbing than etherization. Each animal was at once bled from the inferior vena cava and the autopsy performed immediately. The details of our method of post-mortem examination are elsewhere described (2). The organs weighed were: the gastrointestinal mass, the heart, the liver, the kidneys, the spleen, the brain, the thyroid, the parathyroids, the hypophysis, the suprarenals, the pineal, the thymus, the testicles, the posterior axillary and popliteal lymph nodes, the deep cervical lymph nodes, and the main mass of mesenteric nodes. In order to avoid drying after removal from the body, the small organs were placed in Petri dishes and kept moist with physiological salt solution. Before weighing the excess fluid was removed by absorption on filter paper.

The records kept included the source of each rabbit, the date of receipt, an age grouping, a breed or type classification, a weight chart, and a record of the general physical condition of the animal during the period of observation.

The statistical analysis of the organ weights has included both actual and relative weights, that is, the weight of organs per kilo of net body weight.

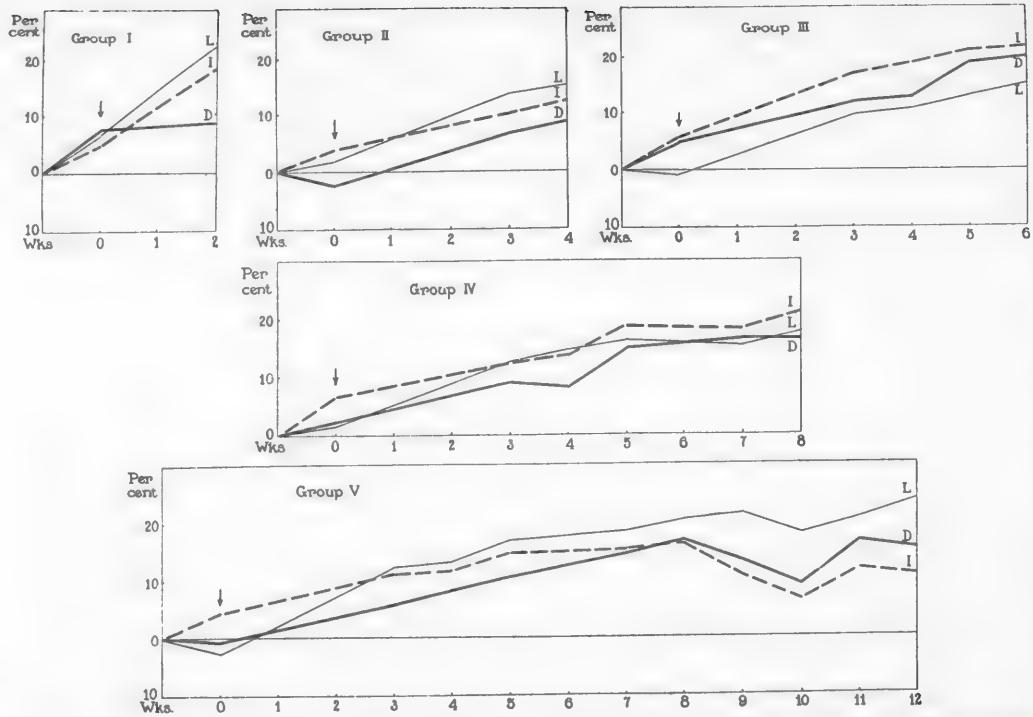
No animal has been omitted because of any abnormality detected either during life or at autopsy, and the figures, as given, include all data irrespective of whether an organ was normal or pathological. Certain groups contain one or more goiters and in these instances additional figures for the thyroid are given from which these weights have been omitted. Similar supplementary figures are given for the deep cervical lymph nodes in those cases in which very large nodes were associated with a purulent infection of the middle ear or cranial sinuses.

RESULTS.

The clinical observations on the 150 rabbits comprising the experiment were made over a period of 6 to 16 weeks, reckoning from the time the animals were first brought to the laboratory; while the dura-

1925. These observations are not included in the present paper because of the introduction of a number of factors not uniform with those reported. Much of the data from this additional experiment, however, is in general conformity with that of the first.

[2] No outside control rabbits are available for the last group of experimental animals. We have, however, included the results obtained from 20 normal rabbits killed and examined on March 12, midway between the time when the fourth and fifth experimental groups were examined. These animals had been caged indoors for 1 to 4 weeks.



TEXT-FIG. 1. Percentage deviations of mean body weights from the mean weights on admission. The number of weeks refers to the time the groups of rabbits had been under conditions of constant light or of constant darkness.

Light groups ——————
Dark groups ━━━━━━
Control groups ━ ━ ━

tion of the experiment, that is, of exposure to constant illumination or to constant exclusion of light, was 2 to 12 weeks. The physical state of all rabbits at the beginning of the experiment was excellent and it continued to be so in the case of the animals in the light room and those in the unaltered or control room. Although the same was generally true for the rabbits caged under conditions of constant darkness there were some indications that this environment was less favorable.

Evidence for the satisfactory condition of the rabbits is furnished by body weight curves (Text-fig. 1) in which the average or mean value for each group of 10 animals is used. The changes in weight are expressed in percentage deviations from the average weight of the group on admission. The curves show that, on the whole, each group of rabbits gained in weight steadily and fairly uniformly. In Groups I and II (2 and 4 weeks exposure) the light room animals increased in weight more than the "inside controls," while those in the dark room gained the least. In Group III (6 weeks exposure) the inside controls and the dark room animals gained more than the light room rabbits. In Group IV (8 weeks exposure) the gain of all three sets approximated each other with a tendency of those in the dark room to gain the least and the inside controls to gain the most; the light room rabbits of this group showed a greater increase in weight during the first weeks than those in the dark room but toward the end of the experiment the two curves are practically identical. In the last group (12 weeks exposure) the greatest increase in body weight was made by the group of light room rabbits, while the inside controls gained the least. The dark room animals showed the smallest gains for the first 7 weeks, but during the last month they slightly exceeded in this particular the inside controls. It should be mentioned that this group of inside controls was not entirely comparable to the others in that it contained three older and larger rabbits. During the first 2 months, these animals gained slightly in weight but in the subsequent month lost weight, which largely contributed to the disproportionate drop in the average body weight of the group during the latter part of the experiment.

Reference to the curves brings out the fact that, except with Group I, the mean body weights of the various groups at the time the rabbits were placed in the light and dark rooms were not uniform. That is,

from the time of admission to the beginning of the experiment, some groups gained less weight than others, while some actually lost weight. A more accurate idea of the effect of the experimental environments upon general physical conditions as indicated by the criterion of body weight is obtained by comparing the final weight values with those at the beginning of the experimental period (Table I). All the control groups showed an increase in weight; the gain of the fifth group was much smaller than the others owing, as has been mentioned above, to the irregularity of the group in respect to the age and size of the animals. The greatest gains in weight were made by the rabbits

TABLE I.

Increase in Mean Body Weight during Experimental Period.

	Initial weights			Final weights			Actual and percentage gain					
	Light room	Dark room	Controls	Light room	Dark room	Controls	Light room		Dark room		Controls	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	gm.	per cent	gm.	per cent
Group I	2200	2125	2198	2528	2190	2433	328	14.9	65	3.1	235	10.7
Group II	2178	2200	2095	2460	2450	2350	282	13.0	250	11.4	255	12.2
Group III	1995	2125	2225	2310	2415	2570	315	15.8	290	13.6	345	15.5
Group IV	2100	2225	2333	2433	2530	2653	333	15.9	305	13.7	320	13.7
Group V	2045	2035	2380	2603	2370	2530	558	27.3	335	16.5	150	6.3
Mean	2104	2142	2246	2467	2391	2507	363	17.3	249	11.6	261	11.6

kept in the light room, and this was true of all five groups. The dark room groups gained less than those in the light room and in the case of the first three groups, less than the inside controls. The percentage increase of the fourth groups of dark room and inside control animals was the same. The fifth group in the dark room showed a much greater increase than the inside controls due largely to the disproportionately high value of the latter group at the beginning of the experiment.

It should be mentioned, in connection with the body weight observations, that each rabbit was given all the food he would consume with the idea of supplying optimum conditions for the maintenance of a

good state of nutrition. The appetite of the rabbits in the light room was noticeably increased, especially during the first 6 weeks of the experiment, and it was found that they ate more food than the controls. The appetite of the dark room rabbits, on the other hand, showed no apparent deviation from the normal.

It is also of interest to note that the general behavior of the rabbits was not apparently disturbed by living in an environment of constant light. They did not try to avoid looking at the arcs and lamps and, as far as could be determined, slept as usual. During the first weeks of the experiment the animals seemed more alert and lively but this condition did not persist. On the other hand, there was nothing in the behavior of the rabbits in the dark room that was peculiar to this group of animals.

The health of the animals was, in general, excellent. Few instances of spontaneous disease developed during the period of observation that were recognizable clinically.

There were 2 cases of ear canker, 1 each among the light room and inside control rabbits; 1 instance of a small, encapsulated, subcutaneous abscess in an inside control animal, and 3 cases of clinical snuffles, all among the inside controls.

It was found by postmortem examination, however, that 59.3 per cent of the rabbits in these groups (light room 60 per cent, dark room 58 per cent, inside controls 60 per cent) showed gross lesions of some kind but in only a few cases were these more than slight or moderate in degree.

The great majority of the lesions, which were distributed fairly uniformly among the subgroups, were scarring of the kidneys and liver, but there was 1 example of a marked chronic coccidiosis of the liver with recent lesions, and 20 instances of a purulent infection of the cranial sinuses, middle ear, or mastoids. Other conditions noted included slight degenerations of the aorta, verminous infections of the intestinal tract, coccidial cysts of the abdominal cavity not associated with visible lesions of the liver, 1 case of a healed pulmonary abscess, 1 instance of a healing abscess of the myocardium and another of hypertrophy of the right ventricle of undetermined origin, 1 case of marked malformation of the kidneys, 1 of a parathyroid cyst, 2 axillary lymph node cysts, and 2 instances of hydrocephalus.

As a further indication of the health of the animal the condition of body fat was noted at autopsy in such general terms as excessive,

abundant, moderate, scant, or none. The body fat was either scanty or practically absent in only 13 rabbits (8.06 per cent) distributed as follows: 3 in the light, 7 in the dark, and 3 in the inside control groups respectively.

A number of gross lesions were found at autopsy in all save 2 of these 13 rabbits.

Of the 3 light room rabbits, there were 2 with active lesions of the kidneys, 1 with a purulent inflammation of both mastoids, and all 3 showed healed or inactive coccidiosis of the liver of slight degree. Among the 7 dark room rabbits, there were 5 with kidney lesions, 1 of which was active and 4 inactive with varying degrees of scarring; there was 1 instance of an active and 1 of a slight and inactive coccidiosis, and 1 of a purulent exudate in the nasal passages and mastoids. Among the 3 inside control rabbits, 2 showed kidney lesions, of which 1 was active and the other inactive with marked scarring; 1 animal had a slight active and 1 a slight inactive coccidiosis, and another had a purulent inflammation of the nasal passages and cranial sinuses. There was some degree of thymic mass depletion in 9 and an enlargement of the spleen in 10 of these 13 rabbits.

The 40 rabbits comprising the four groups of outside controls (recently acquired animals) were clinically free from any evidence of disease but there was practically the same incidence of visible lesions at autopsy as in the other groups, that is, 57.5 per cent.

In the case of 20 normal rabbits killed on March 12, 1925, midway between the fourth and fifth experimental groups, the incidence of postmortem lesions was 60 per cent. The types of lesion did not differ from those found in the light, dark, and inside control rabbits, the majority being slight or moderate and principally affecting the same organs. Among the outside controls there were 2 cases of a marked active coccidiosis of the liver and 5 instances of a moderate or marked scarring of the kidneys. In the March group, the incidence of active coccidiosis of considerable extent was higher—5 of the 20 rabbits showing this condition; in 2 animals there was marked scarring of the kidneys. In none of the outside controls was there any purulent inflammation about the head, while 2 of the March normals showed this condition. The body fat of 3 outside control rabbits was scanty. In each animal the thymic mass was small and the spleen enlarged, but only 1 showed gross lesions of probable significance, namely, a possibly active lesion of the kidneys and an active coccidiosis of the liver. A similar state of body fat occurred in 6 of the 20 March normals, 4 of which showed a small thymus and 3 of these a slightly enlarged spleen. There was 1 instance of an active and another of an inactive coccidiosis, 1 of a purulent inflammation of the mastoids, while in 3 rabbits no gross lesions were found. The occurrence of relatively little

fat in rabbits which presumably have not been living a sedentary life and have not necessarily been constantly provided with abundant food is obviously of less significance as an index of physical condition than it is in rabbits living a caged existence with an ample food supply.

DISCUSSION.

The health of the animals employed in an experiment of this type is of importance first, from the point of view of the general reaction to artificial environments, and second, because of the influence of physical condition upon the weights of organs. The rabbits comprising this experiment were all in apparent good health throughout the period of observation, such spontaneous disease as could be recognized clinically appearing unimportant.

Body weight observations have been used as an index to the effects of constant illumination or exclusion of light upon the general physical condition. There were five groups of 10 rabbits kept under each of these environmental conditions for 2, 4, 6, 8, and 12 weeks respectively. All groups gained in weight during these periods, but the greatest gains were made by the rabbits exposed to constant light. While there was also an increase in body weight of the groups kept in constant darkness it was less pronounced and more irregular than that of the groups in constant light and, on the whole, than that of the control rabbits kept in an ordinary animal room, a fact which suggests that the exclusion of light was relatively unfavorable.

At the end of the experimental period, all the rabbits appeared to be in good condition, but postmortem examination showed that the body fat of several was relatively scanty. The distribution of these animals, 3 in the light, 3 in the control, and 7 in the dark room groups, suggests that some impairment of nutrition may be related to prolonged caging in an absence of light. This is further suggested by the fact that 4 of the 7 dark room rabbits were of the group which had been kept in this environment for 12 weeks. Gross lesions of some kind were found in 11 of the 13 rabbits with scant body fat, those of the kidneys appearing most significant. The kidneys were affected in 9 instances and in 4 of these the lesions appeared active, the organs being enlarged, soft, opaque, and either markedly congested or yellow. To what extent inactive lesions of the kidneys, with moderate or

marked scarring, are associated with scant body fat is not known, but such was the condition in 5 rabbits. On the other hand, moderate or marked renal scarring is not necessarily accompanied by scant body fat, for there were 19 other rabbits among the 150 experimental animals with such lesions but with no evident fat reduction.

The thymic mass in the rabbit has been found to be a fairly delicate indicator of general physical condition in that its size, consistency, and the amount of associated fat are roughly proportional to the state of nutrition of the animal. Among the 13 rabbits of the experimental series which showed a scant amount of body fat, the thymic mass was considered to be depleted to a greater or less extent in 9 animals, 5 of which were in the dark room groups.

Enlargement of the spleen was frequently seen in the entire series of rabbits and no relationship could be made out between its occurrence and the presence or character of gross lesions, or the condition of the body fat.

At postmortem examination it was found that visible lesions of some kind were present in 59.3 per cent of the 150 rabbits caged under conditions of constant illumination, of constant darkness, and of an ordinary animal room. Lesions of a similar type and distribution were found in 57.5 per cent of one group of 40, and in 60 per cent of a group of 20 rabbits recently brought to the laboratory. These figures are higher that those of two groups of 350 and 295 normal rabbits examined during $3\frac{1}{2}$ years, namely, 40.9 and 53.2 per cent (3). While the larger number of animals in these two series suggests a possible explanation for these differences, the uniform incidence of lesions among the four major groups of this experiment indicates a higher proportion of pathological change in the rabbit stock at this particular time. The state of the lesions found in the experimental animals did not suggest that the conditions of constant light or of constant darkness were associated with heightened activity of the pathological process.

It has been found from the observations of the two large series of normal rabbits referred to, that as long as the animals remain in good health the values obtained for organ weights of animals with lesions do not differ materially from those of animals that are entirely free from lesions (3, 4). There is a slight tendency toward an increase

in organ weight as one passes from rabbits with slight or moderate lesions to those with marked lesions, particularly in relation to the activity of such conditions. The number of marked or active lesions was extremely few in this series of rabbits, and it has been found by actual determination that these conditions have not essentially affected the mean organ values employed in analyzing the experimental results obtained, except in certain particular instances, as in the case of the deep cervical lymph nodes, a marked enlargement of which is associated with a purulent infection of the cranial sinuses. The effect of an impaired state of health upon organ weight does not have to be considered in the present connection as all the rabbits of the experiment were in good physical condition during the period of observation.

SUMMARY AND CONCLUSIONS.

A group of 50 normal male rabbits kept under conditions of constant light that had none of the shorter ultra-violet rays and another group kept in constant darkness for 2 to 12 weeks were observed clinically and subjected to postmortem examination for the purpose of determining the effect of these environmental conditions upon general body health and the weights of organs. A similar group of 50 rabbits caged in an ordinary animal room for the same period, and two groups of 40 and 20 rabbits respectively, which had recently been brought into the laboratory, served as controls.

It was found that the general health of the rabbits was not impaired by the artificial light or the exclusion of light. The gain in body weight which occurred in all groups was especially marked in the case of those kept under conditions of constant light.

The incidence of spontaneous disease recognizable clinically during the experiment was extremely low and of a mild character and did not obviously disturb the health of the animal. It was found at postmortem examination, on the other hand, that 59.3 per cent of the rabbits caged indoors, that is in the light, dark, or unaltered rooms, and 58.3 per cent of those recently brought to the laboratory had visible lesions of some kind. The great majority of these lesions, however, were of a slight grade, and none appeared to have any deleterious effects upon the general physical state of the animals.

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EFFECTS OF LIGHT ON NORMAL RABBITS, WITH ESPECIAL REFERENCE TO THE ORGANIC REACTION.

II. Organ Weights.

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In Paper I of this series (1), it was stated that the purpose of our investigation was the testing by experimental means, of whether there is a relationship between the nature or character of the light environment, on the one hand, and the physical state and functional activities of the animal organism, on the other. The methods employed and the general clinical observations and postmortem findings have been described. The present paper is concerned with the effects of light upon organ weights.

RESULTS.

The weights of the organs are presented in tabular form. The actual weights are given in Table I and the relative weights, or the weights per kilo of net body weight, in Table II. In both tables the order of organs and of animal groups is the same. For comparison, observations obtained from a series of 350 normal rabbits are given first (2).[1] Then follow the results of this experiment in the order of light and dark room animals and inside and outside controls. With the exception of the outside controls, these major divisions are divided into five groups, comprising 10 rabbits each, and corresponding to the 2, 4, 6, 8, and 12 week periods of exposure to the environments of the experiment. There are only four groups of outside controls. Fi-

[1] In the case of the gross body weight, the kidneys (actual), testicles (relative), suprarenals (actual), and axillary lymph nodes (actual), the figures given for the 350 normal rabbits differ in a few respects from those in the original paper. The changes represent corrections of slight errors in the original figures.

nally, results are given for 20 normal rabbits examined on March 12, 1925, midway in time between the fourth and fifth experimental groups.

COMMENT.

This portion of the paper is concerned mainly with the data obtained. An analysis of the organ weights contained in Tables I and II will be taken up in Paper III (3).

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TABLE I.

Results for Body and Actual Organ Weights According to Groups.

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Gross body weight	N I	2265	1400	3500	352.8	237.90	15.57
	L 1	2528	2200	3325	323.7	218.33	12.81
	2	2460	1850	3000	375.5	253.28	15.26
	3	2310	2050	2650	194.0	130.85	8.40
	4	2433	2125	2875	247.5	176.94	10.18
	5	2603	2100	3225	373.5	251.93	14.35
	D 1	2188	1825	2475	210.7	142.10	9.63
	2	2460	2200	2750	179.3	120.90	7.28
	3	2415	2150	2975	229.7	154.93	9.51
	4	2530	2275	3000	236.3	159.40	9.33
	5	2358	1925	2825	309.2	208.60	13.11
	I 1	2478	2150	3050	248.1	167.34	10.01
	2	2350	2175	2650	137.8	92.95	5.86
	3	2570	2350	2775	147.3	99.35	5.73
	4	2618	2275	2950	249.5	168.30	9.53
	5	2545	2075	3100	353.9	238.70	13.90
	O 1	2235	1800	2600	246.2	166.06	11.02
	2	2440	2050	3200	321.1	216.58	13.16
	3	2313	1950	2950	313.5	211.46	13.56
	4	2158	1800	2725	240.3	162.08	11.14
	M	2024	1725	2450	208.9	140.93	10.32
Gastrointestinal mass	N I	416.4	115	710	80.9	54.5	19.42
	L 1	454.0	340	570	66.1	45.58	14.56
	2	413.0	327	505	59.1	39.86	14.30
	3	325.0	230	415	63.2	42.63	19.45
	4	338.0	260	402	44.7	30.15	13.23
	5	415.0	325	562	80.2	50.06	19.31
	D 1	432.0	375	485	38.8	26.17	8.99
	2	473.0	325	640	89.1	60.06	18.84
	3	421.0	295	580	76.4	51.53	18.14
	4	428.0	305	495	48.8	32.9	11.40
	5	427.0	305	575	83.1	56.05	19.45
	I 1	469.0	335	610	81.9	53.24	17.46
	2	438.0	305	555	82.1	53.35	18.74
	3	428.0	337	532	56.4	38.04	13.18
	4	432.0	305	565	77.2	52.1	17.87
	5	434.0	360	555	57.6	38.8	13.28
	O 1	501.0	365	695	91.4	61.65	18.24
	2	538.0	400	740	104.5	70.49	19.42
	3	447.0	355	507	33.8	22.8	7.56
	4	446.0	345	570	67.5	45.53	15.14
	M	383.0	195	500	64.4	43.42	16.81

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Net body weight	N I	1848.5	1150	3010	323.3	218.0	17.49
	L 1	2074.0	1780	2755	287.6	193.98	13.87
	2	2048.0	1450	2585	358.6	241.86	17.52
	3	1985.0	1760	2235	157.7	106.37	7.95
	4	2094.0	1805	2500	232.0	156.48	11.08
	5	2188.0	1756	2730	304.9	205.66	13.93
	D 1	1756.0	1440	2035	210.8	142.20	12.00
	2	1988.0	1795	2260	150.8	101.71	7.58
	3	1994.0	1765	2395	175.4	118.31	8.79
	4	2102.0	1860	2505	213.4	143.94	10.16
	5	1930.0	1613	2415	267.0	180.10	13.83
	I 1	2009.0	1695	2440	198.1	133.62	9.86
	2	1913.0	1765	2175	126.8	85.53	6.63
	3	2142.0	1885	2350	124.4	83.91	5.81
	4	2194.0	1955	2500	198.1	133.60	9.03
	5	2112.0	1715	2660	327.9	221.20	15.53
	O 1	1718.0	1395	2135	247.5	166.94	14.41
	2	1902.0	1477	2505	306.9	207.00	16.14
	3	1866.0	1505	2485	305.2	205.86	16.36
	4	1711.0	1455	2190	211.9	142.93	12.38
	M	1641.0	1380	2020	184.7	124.59	11.26
Heart	N I	5.26	3.20	10.18	0.96	0.64	18.23
	L 1	5.90	4.86	7.70	0.898	0.606	15.22
	2	5.97	4.40	9.24	1.29	0.87	21.61
	3	5.38	4.50	6.70	0.719	0.485	13.34
	4	5.22	4.25	6.42	0.706	0.476	13.52
	5	6.02	4.85	7.85	0.90	0.61	15.02
	D 1	5.40	4.40	6.54	0.69	0.47	12.78
	2	5.55	4.67	6.82	0.71	0.48	12.79
	3	5.33	4.50	6.55	0.58	0.39	10.84
	4	5.52	4.45	6.85	0.72	0.48	12.97
	5	5.30	4.50	6.65	0.68	0.46	12.90
	I 1	5.81	4.65	6.64	0.64	0.43	10.95
	2	5.66	4.45	8.05	0.96	0.65	16.93
	3	5.54	4.61	6.95	0.65	0.44	11.81
	4	6.19	5.10	7.65	0.882	0.594	14.25
	5	5.86	4.30	7.43	0.77	0.52	13.05
	O 1	5.40	3.86	7.30	0.89	0.60	16.48
	2	6.05	4.63	8.00	0.90	0.61	14.86
	3	5.76	3.95	7.50	1.07	0.72	18.58
	4	5.15	4.40	7.40	0.90	0.61	17.50
	M	4.87	3.55	6.55	0.604	0.41	12.39

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Liver	N I	84.3	50.0	150.0	19.7	13.3	23.39
	L 1	114.4	95.0	140.0	12.46	6.88	10.88
	2	70.6	57.0	82.0	7.78	5.25	11.02
	3	79.8	67.0	100.0	9.67	6.52	12.12
	4	74.7	60.0	100.0	12.80	8.63	17.14
	5	77.3	55.0	105.0	13.92	9.39	18.01
	D 1	99.5	78.0	111.0	8.86	5.98	8.90
	2	97.2	65.0	122.0	14.50	9.78	14.91
	3	101.8	80.0	135.0	18.7	12.6	18.36
	4	109.0	73.0	155.0	23.08	15.57	21.17
	5	110.9	85.0	145.0	17.8	12.01	16.05
	I 1	92.6	74.0	115.0	11.99	8.09	12.95
	2	99.1	85.0	118.0	10.90	7.35	10.99
	3	80.1	63.0	95.0	9.9	6.68	12.46
	4	95.4	65.0	127.0	18.45	12.24	19.34
	5	94.7	67.0	120.0	16.86	11.37	17.81
	O 1	106.2	57.0	185.0	32.7	22.06	30.89
	2	85.6	67.0	122.0	15.1	10.19	17.64
	3	73.3	55.0	100.0	11.7	7.89	15.96
	4	80.6	55.0	120.0	19.7	13.29	24.44
	M	89.9	55.0	120.0	15.04	10.15	16.73
Kidneys	N I	12.7	7.15	22.73	2.14	1.44	16.85
	L 1	15.44	13.20	18.50	1.84	1.24	11.92
	2	13.59	9.55	21.25	3.11	2.10	22.88
	3	12.16	10.92	13.25	0.797	0.54	6.55
	4	12.55	11.05	15.60	1.40	0.95	11.18
	5	13.21	8.80	16.68	1.84	1.24	13.92
	D 1	14.00	10.85	17.80	2.13	1.44	15.21
	2	14.60	11.40	17.30	1.99	1.34	13.63
	3	14.28	11.80	16.55	1.44	0.97	10.04
	4	13.86	10.80	17.04	1.92	1.29	13.85
	5	12.61	9.20	14.42	1.58	1.07	12.52
	I 1	15.25	13.45	17.67	1.19	0.80	7.80
	2	14.01	10.37	16.90	1.91	1.29	13.63
	3	14.05	11.90	16.34	1.27	0.86	9.04
	4	13.89	11.60	19.40	2.33	1.57	16.78
	5	13.51	9.25	19.97	2.84	1.91	21.01
	O 1	13.46	9.65	17.56	2.26	1.52	16.80
	2	14.25	11.32	17.85	1.98	1.34	13.89
	3	13.80	11.50	19.18	2.24	1.51	16.23
	4	12.98	10.00	15.10	1.47	0.99	11.33
	M	13.20	10.90	16.70	1.53	1.03	11.56

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Spleen	N I	0.990	0.08	3.20	0.436	0.294	44.04
	L 1	1.344	0.89	2.28	0.425	0.287	31.62
	2	1.209	0.64	1.90	0.408	0.275	33.75
	3	1.005	0.40	2.18	0.512	0.345	50.95
	4	0.978	0.355	2.035	0.421	0.284	44.06
	5	1.270	0.095	2.35	0.658	0.444	51.81
	D 1	1.314	0.50	1.92	0.405	0.273	30.82
	2	1.154	0.745	1.705	0.378	0.255	32.75
	3	1.400	0.80	2.82	0.547	0.369	39.07
	4	1.021	0.575	1.535	0.286	0.193	28.01
	5	1.195	0.65	2.285	0.510	0.344	42.67
	I 1	1.594	0.88	3.33	0.719	0.485	44.48
	2	1.273	0.59	2.575	0.525	0.354	41.24
	3	1.112	0.715	2.15	0.417	0.281	37.50
	4	1.108	0.56	1.715	0.331	0.223	29.84
	5	1.309	0.87	2.685	0.537	0.362	41.02
	O 1	1.193	0.71	1.67	0.270	0.182	22.63
	2	1.104	0.55	1.94	0.374	0.252	33.88
	3	1.433	0.56	3.00	0.665	0.449	46.41
	4	1.245	0.53	1.535	0.274	0.185	22.01
	M	1.110	0.47	2.17	0.462	0.312	41.60
Thymus	N I	2.210	0.45	5.90	0.848	0.572	38.37
	L 1	2.865	1.94	4.38	0.881	0.594	30.75
	2	2.378	1.54	3.70	0.759	0.512	31.92
	3	2.182	1.54	3.23	0.421	0.284	19.27
	4	2.671	1.86	4.25	0.687	0.464	25.73
	5	3.136	1.85	4.97	1.043	0.704	33.26
	D 1	2.527	0.91	3.78	0.784	0.529	31.03
	2	2.401	1.54	3.55	0.659	0.444	27.44
	3	2.350	0.82	3.40	0.680	0.459	28.93
	4	2.105	1.30	2.92	0.471	0.318	22.37
	5	2.213	0.90	3.90	0.959	0.647	43.33
	I 1	3.328	1.70	6.30	1.493	1.007	44.86
	2	2.577	1.26	3.50	0.655	0.442	25.42
	3	2.338	1.43	3.05	0.466	0.314	19.93
	4	2.464	0.63	4.10	0.937	0.632	38.04
	5	1.967	1.30	3.15	0.622	0.420	31.62
	O 1	1.670	0.97	3.85	0.906	0.611	54.25
	2	2.039	0.73	3.55	0.931	0.628	45.66
	3	2.028	1.17	3.00	0.562	0.379	27.71
	4	1.666	0.95	3.17	0.677	0.457	40.64
	M	2.260	0.80	3.50	0.851	0.574	37.65

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Testicles	N I	4.86	0.81	9.12	1.63	1.10	33.54
	L 1	5.08	4.40	6.33	0.57	0.39	11.20
	2	5.65	3.55	8.50	1.38	0.93	24.42
	3	5.07	4.15	6.70	0.68	0.46	13.31
	4	5.25	3.70	6.89	0.99	0.67	18.86
	5	5.23	4.05	6.43	0.67	0.45	12.72
	D 1	4.92	3.73	7.15	0.995	0.67	20.22
	2	5.39	3.25	6.90	0.904	0.61	16.77
	3	5.77	4.30	7.17	0.87	0.59	15.06
	4	5.31	3.90	6.60	0.94	0.63	17.70
	5	4.57	2.55	6.20	1.07	0.72	23.41
	I 1	5.50	3.90	7.72	1.04	0.70	18.91
	2	5.23	3.88	6.88	1.10	0.74	21.05
	3	6.06	4.98	8.05	0.92	0.62	15.13
	4	5.88	4.55	7.35	0.97	0.66	16.51
	5	4.81	3.28	6.90	0.99	0.67	20.58
	O 1	5.79	1.77	9.01	2.23	1.50	38.51
	2	5.13	3.13	7.10	1.51	1.02	29.44
	3	5.05	1.75	8.69	2.10	1.42	41.58
	4	4.53	3.40	6.30	0.86	0.58	18.92
	M	4.27	2.75	7.20	1.08	0.73	25.32
Brain	N I	9.31	7.42	12.03	0.78	0.526	8.37
	L 1	9.34	8.10	10.00	0.59	0.40	6.28
	2	9.56	8.65	10.45	0.49	0.33	5.15
	3	8.67	7.51	9.75	0.77	0.52	8.99
	4	8.84	8.15	9.70	0.51	0.34	5.74
	5	9.73	8.05	10.90	0.81	0.54	8.28
	D 1	9.60	8.75	10.50	0.62	0.42	6.48
	2	9.22	8.00	10.43	0.71	0.48	7.65
	3	9.46	8.40	10.39	0.674	0.454	7.12
	4	9.10	8.10	10.75	0.80	0.54	8.80
	5	9.08	7.35	10.00	0.73	0.49	8.05
	I 1	9.49	7.57	11.00	0.86	0.58	9.10
	2	9.21	8.00	10.24	0.68	0.46	7.33
	3	9.49	7.90	11.33	0.94	0.63	9.90
	4	9.24	8.65	9.82	0.373	0.251	4.04
	5	9.35	8.25	10.20	0.596	0.40	6.38
	O 1	9.27	8.00	10.00	0.64	0.43	6.87
	2	9.62	8.07	11.30	0.92	0.62	9.58
	3	9.39	8.19	10.45	0.58	0.39	6.16
	4	8.90	8.10	9.45	0.46	0.31	5.15
	M	8.76	8.10	10.05	0.60	0.40	6.84

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Thyroid	N I	0.23048	0.085	1.570	0.16209	0.10933	70.33
	L 1	0.2055	0.120	0.305	0.06109	0.04121	29.72
	2	0.1835	0.120	0.260	0.03772	0.02544	20.56
	3	0.1950	0.150	0.280	0.0344	0.0232	17.64
	4	0.2600	0.155	0.375	0.0629	0.04243	24.19
	5	0.2550	0.160	0.355	0.0668	0.04506	26.20
	D 1	0.1880	0.125	0.330	0.0536	0.0362	28.51
	2	0.2219	0.150	0.330	0.0483	0.0326	21.76
	3	0.2467	0.185	0.315	0.0443	0.0299	17.95
	4	0.2395	0.160	0.340	0.0536	0.0362	22.37
	5	0.2545	0.160	0.810	0.1866	0.1259	73.32
		(0.1928)	(0.160)	(0.245)	(0.0252)	(0.017)	(13.07)
	I 1	0.2459	0.165	0.380	0.06304	0.04252	25.64
	2	0.2657	0.163	0.397	0.0638	0.0430	24.01
	3	0.3180	0.180	0.512	0.0899	0.0606	28.27
		(0.2964)	(0.180)	(0.385)	(0.06608)	(0.0445)	(22.94)
	4	0.4115	0.208	0.935	0.2424	0.1635	58.90
		(0.2657)	(0.208)	(0.310)	(0.0426)	(0.0287)	(16.02)
	5	0.3082	0.190	0.390	0.0695	0.0469	22.55
	O 1	0.2196	0.107	0.520	0.1089	0.0735	49.59
		(0.1862)	(0.107)	(0.255)	(0.0451)	(0.0304)	(24.22)
	2	0.1880	0.115	0.280	0.0479	0.0323	25.48
	3	0.1852	0.115	0.410	0.0439	0.0296	23.70
	4	0.2540	0.110	0.480	0.1231	0.0830	48.46
		(0.2289)	(0.110)	(0.410)	(0.1025)	(0.0690)	44.77
	M	0.2622	0.120	1.730	0.3403	0.2296	129.70
		(0.1852)	(0.120)	(0.340)	(0.0510)	(0.0344)	(27.54)
Parathyroids	N I	0.01256	0.002	0.035	0.00486	0.00328	38.69
	L 1	0.01260	0.008	0.015	0.00220	0.00148	17.46
	2	0.01020	0.006	0.019	0.00329	0.00222	32.24
	3	0.00970	0.006	0.016	0.00290	0.00196	29.90
	4	0.00950	0.004	0.013	0.00253	0.00171	26.63
	5	0.01170	0.007	0.020	0.00387	0.00261	33.08
	D 1	0.01300	0.007	0.018	0.00355	0.00239	27.30
	2	0.01080	0.005	0.015	0.00309	0.00208	28.61
	3	0.01310	0.008	0.018	0.00270	0.00182	20.61
	4	0.01160	0.006	0.022	0.00480	0.00324	41.37
	5	0.01330	0.005	0.024	0.00655	0.00442	49.24
	I 1	0.01200	0.009	0.020	0.00328	0.00221	26.89
	2	0.01260	0.007	0.022	0.00412	0.00278	32.70
	3	0.01310	0.008	0.025	0.00452	0.00305	34.50
	4	0.01222	0.006	0.019	0.00394	0.00266	32.22
	5	0.01840	0.013	0.027	0.00500	0.00338	27.20

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Parathyroids —Continued	O 1	0.01460	0.008	0.033	0.00687	0.00463	47.05
	2	0.01230	0.007	0.027	0.00558	0.00376	45.37
	3	0.01170	0.004	0.016	0.00304	0.00206	25.98
	4	0.00850	0.003	0.014	0.00323	0.00218	38.00
	M	0.01070	0.006	0.017	0.00357	0.00241	33.32
Hypophysis	N I	0.0280	0.015	0.044	0.00517	0.00349	18.46
	L 1	0.0272	0.017	0.045	0.00802	0.00541	29.49
	2	0.0291	0.018	0.045	0.00664	0.00448	22.82
	3	0.0248	0.022	0.028	0.00183	0.00123	7.38
	4	0.0253	0.019	0.032	0.00349	0.00235	13.80
	5	0.0251	0.020	0.033	0.00353	0.00238	14.06
	D 1	0.0276	0.022	0.039	0.00471	0.00318	17.07
	2	0.0278	0.023	0.042	0.00526	0.00355	18.92
	3	0.0293	0.025	0.035	0.0034	0.00229	11.60
	4	0.0237	0.016	0.038	0.0058	0.00391	24.47
	5	0.0263	0.020	0.031	0.00363	0.00245	13.80
	I 1	0.0281	0.021	0.037	0.00441	0.00298	15.69
	2	0.0306	0.022	0.040	0.00574	0.00387	18.76
	3	0.0283	0.022	0.037	0.00392	0.00264	13.85
	4	0.0295	0.022	0.038	0.00554	0.00373	18.77
	5	0.0291	0.022	0.038	0.00505	0.00341	17.35
	O 1	0.0282	0.023	0.037	0.00376	0.00254	13.33
	2	0.0276	0.018	0.037	0.00514	0.00347	18.62
	3	0.0279	0.015	0.040	0.00665	0.00449	23.84
	4	0.0254	0.020	0.032	0.00366	0.00247	14.41
	M	0.0279	0.020	0.035	0.0038	0.00256	13.64
Suprarenals	N I	0.3858	0.115	1.050	0.1546	0.10428	40.07
	L 1	0.373	0.145	0.630	0.1383	0.09328	37.13
	2	0.478	0.280	1.112	0.2289	0.15439	47.89
	3	0.507	0.270	0.950	0.2198	0.14826	43.35
	4	0.425	0.346	0.517	0.0576	0.03885	13.55
	5	0.471	0.310	0.620	0.0939	0.06334	19.94
	D 1	0.317	0.180	0.485	0.0843	0.0569	26.55
	2	0.435	0.230	0.580	0.1091	0.0736	25.08
	3	0.395	0.260	0.650	0.1384	0.0934	35.03
	4	0.438	0.270	0.680	0.1330	0.0897	30.40
	5	0.498	0.250	0.940	0.2151	0.14508	43.20
	I 1	0.399	0.265	0.650	0.1107	0.0747	27.74
	2	0.426	0.180	0.865	0.1935	0.1305	45.42
	3	0.394	0.205	0.605	0.1088	0.0734	27.59
	4	0.487	0.255	0.750	0.1580	0.1063	32.39
	5	0.514	0.280	0.885	0.2180	0.1457	42.43

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Suprarenals—Continued	O 1	0.413	0.250	0.760	0.1627	0.1097	39.29
	2	0.430	0.198	0.720	0.1921	0.1296	44.67
	3	0.419	0.192	0.710	0.1593	0.1075	38.01
	4	0.333	0.145	0.640	0.1604	0.1082	48.17
	M	0.269	0.138	0.530	0.1018	0.0687	37.83
Pineal	N I	0.01609	0.005	0.030	0.00429	0.00289	26.66
	L 1	0.01330	0.010	0.016	0.00205	0.00138	15.41
	2	0.01240	0.008	0.016	0.00280	0.00189	22.58
	3	0.01290	0.006	0.017	0.00401	0.00271	31.09
	4	0.01260	0.007	0.019	0.00349	0.00235	27.38
	5	0.01360	0.011	0.017	0.00174	0.00107	12.79
	D 1	0.01430	0.009	0.023	0.00395	0.00266	27.62
	2	0.01270	0.010	0.016	0.00205	0.00138	16.12
	3	0.01620	0.008	0.022	0.00384	0.00259	23.70
	4	0.01540	0.010	0.023	0.00436	0.00294	28.31
	5	0.01440	0.008	0.021	0.00366	0.00247	25.41
	I 1	0.01510	0.010	0.022	0.00381	0.00257	25.23
	2	0.01580	0.012	0.020	0.00302	0.00204	19.11
	3	0.01450	0.005	0.020	0.00366	0.00245	25.24
	4	0.01450	0.008	0.021	0.00432	0.00291	29.78
	5	0.01690	0.010	0.023	0.00396	0.00267	23.44
	O 1	0.01366	0.010	0.020	0.00380	0.00256	27.74
	2	0.01430	0.011	0.027	0.00447	0.00302	31.25
	3	0.01320	0.005	0.025	0.00563	0.00380	42.65
	4	0.01300	0.008	0.020	0.00434	0.00293	31.08
	M	0.01345	0.005	0.018	0.00347	0.00234	25.80
Popliteal lymph nodes	N I	0.25539	0.085	0.720	0.08241	0.05559	32.27
	L 1	0.273	0.170	0.400	0.0682	0.0460	24.98
	2	0.239	0.180	0.350	0.0482	0.0325	20.17
	3	0.242	0.170	0.350	0.0463	0.03123	19.13
	4	0.236	0.145	0.430	0.0857	0.05781	37.10
	5	0.241	0.145	0.350	0.0585	0.03946	24.27
	D 1	0.277	0.200	0.370	0.0487	0.0329	17.61
	2	0.292	0.220	0.360	0.0498	0.0336	17.08
	3	0.231	0.105	0.350	0.0661	0.0446	28.61
	4	0.248	0.155	0.330	0.0462	0.0312	18.62
	5	0.191	0.100	0.310	0.0666	0.0449	34.86
	I 1	0.313	0.200	0.405	0.0568	0.0383	18.15
	2	0.250	0.150	0.350	0.0576	0.0389	23.04
	3	0.254	0.165	0.420	0.0646	0.0436	25.43
	4	0.251	0.180	0.295	0.0414	0.0279	16.49
	5	0.198	0.090	0.350	0.0682	0.0460	34.43

TABLE I—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Popliteal	O 1	0.181	0.125	0.260	0.0459	0.0310	25.36
lymph	2	0.254	0.135	0.390	0.0691	0.0466	27.21
nodes—	3	0.282	0.190	0.450	0.0664	0.0448	23.55
Continued	4	0.233	0.160	0.410	0.0771	0.0520	32.66
	M	0.242	0.130	0.430	0.0977	0.0659	40.38
Axillary	N I	0.17585	0.040	0.400	0.06141	0.04142	34.92
lymph	L 1	0.17300	0.105	0.240	0.04226	0.02850	24.83
nodes	2	0.14600	0.085	0.300	0.06280	0.04236	43.01
	3	0.15500	0.080	0.305	0.05780	0.03899	37.29
	4	0.14500	0.100	0.235	0.04230	0.02853	29.17
	5	0.14200	0.080	0.220	0.03510	0.02668	24.72
	D 1	0.18750	0.130	0.270	0.03960	0.02670	21.12
	2	0.16200	0.065	0.215	0.04560	0.03080	28.14
	3	0.17000	0.070	0.240	0.05080	0.03430	29.88
	4	0.14280	0.085	0.180	0.03410	0.02300	23.87
	5	0.12650	0.075	0.165	0.03080	0.02080	24.34
	I 1	0.20200	0.095	0.280	0.05140	0.03470	25.45
	2	0.16000	0.125	0.230	0.03200	0.02170	20.06
	3	0.17800	0.105	0.240	0.04960	0.03350	27.92
	4	0.16400	0.085	0.210	0.04240	0.02860	25.83
	5	0.13700	0.100	0.180	0.02840	0.01910	20.73
	O 1	0.12400	0.090	0.170	0.03000	0.02000	24.29
	2	0.13900	0.090	0.205	0.03600	0.02400	25.91
	3	0.20400	0.112	0.365	0.07500	0.05100	36.91
	4	0.16300	0.110	0.265	0.04500	0.03000	27.36
	M	0.16600	0.070	0.485	0.09400	0.06300	56.22
Deep cervical	N I	0.15579	0.035	0.405	0.0732	0.04938	46.98
lymph	L 1	0.13600	0.085	0.182	0.03202	0.02160	23.54
nodes	2	0.12700	0.075	0.235	0.04399	0.02967	34.64
	3	0.14300	0.080	0.235	0.0525	0.03540	36.71
	4	0.10600	0.065	0.165	0.0314	0.02118	29.62
	5	0.23200	0.095	0.550	0.1604	0.10819	69.14
		(0.15400)	(0.095)	(0.250)	(0.0490)	(0.03320)	(31.87)
	D 1	0.16350	0.100	0.220	0.0435	0.02930	26.60
	2	0.18700	0.070	0.380	0.0838	0.05650	44.81
	3	0.15150	0.090	0.350	0.0712	0.04800	46.99
	4	0.11600	0.045	0.260	0.0549	0.03700	47.32
	5	0.13950	0.095	0.380	0.0815	0.05500	58.42
		(0.11300)	(0.095)	(0.140)	(0.0155)	(0.01040)	(13.72)
	I 1	0.19200	0.120	0.460	0.0954	0.06430	49.69
	2	0.14900	0.090	0.190	0.0317	0.02140	21.28
	3	0.13800	0.095	0.215	0.0343	0.02310	24.86

TABLE I—*Concluded.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Deep cervical lymph nodes—Continued	4	0.15200	0.090	0.340	0.0658	0.04440	43.27
		(0.13100)	(0.090)	(0.160)	(0.0210)	(0.01400)	(15.68)
	5	0.22200	0.100	0.540	0.1209	0.08160	54.48
		(0.18700)	(0.100)	(0.295)	(0.0614)	(0.04140	(32.90)
	O 1	0.15000	0.085	0.235	0.0500	0.03400)	32.20
	2	0.15200	0.110	0.195	0.0280	0.01900	18.49
	3	0.15000	0.070	0.285	0.0630	0.04200	41.67
	4	0.15300	0.075	0.360	0.0834	0.05600	54.51
	M	0.14900	0.050	0.505	0.0494	0.03300	33.27
		(0.0120)	(0.050)	(0.220)	(0.0435)	(0.0293)	(36.32)
Mesenteric lymph nodes	N I	3.488	1.34	9.19	1.310	0.880	37.53
	L 1	3.160	1.80	4.25	0.673	0.454	21.30
	2	2.860	1.34	4.50	0.866	0.584	30.28
	3	2.998	2.02	4.50	0.655	0.442	21.85
	4	2.686	2.10	4.45	0.641	0.433	23.87
	5	2.950	1.60	4.60	0.909	0.613	30.48
	D 1	3.219	2.50	4.47	0.639	0.431	19.85
	2	2.950	1.71	3.68	0.674	0.455	22.84
	3	2.950	2.00	5.00	0.808	0.545	27.38
	4	2.680	2.35	3.10	0.206	0.139	7.68
	5	2.460	1.70	3.63	0.640	0.432	26.01
	I 1	3.235	2.20	4.85	0.798	0.538	24.73
	2	2.950	1.85	3.55	0.559	0.377	18.95
	3	3.290	2.45	4.45	0.565	0.381	17.17
	4	3.077	2.00	4.75	0.732	0.493	23.78
	5	3.020	2.00	4.15	0.695	0.468	23.01
	O 1	4.028	1.79	6.17	1.437	0.969	33.12
	2	5.680	2.40	11.47	2.460	1.659	43.31
	3	4.350	2.55	6.75	1.130	0.751	25.59
	4	4.550	3.23	5.75	0.796	0.537	17.50
	M	3.640	2.40	5.65	0.830	0.560	22.80

TABLE II.

Results for Body and Relative Organ Weights According to Groups.

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Heart	N I	2.85	1.95	4.42	0.35	0.24	12.32
	L 1	2.85	2.46	3.31	0.266	0.179	9.33
	2	2.91	2.52	3.57	0.307	0.207	15.50
	3	2.71	2.33	3.24	0.308	0.208	11.37
	4	2.49	2.16	2.74	0.177	0.119	7.11
	5	2.75	2.35	3.05	0.18	0.12	6.44
	D 1	3.09	2.55	3.64	0.32	0.21	10.26
	2	2.80	2.35	3.47	0.38	0.25	13.42
	3	2.66	2.52	2.85	0.11	0.07	4.14
	4	2.63	2.22	3.14	0.30	0.20	11.21
	5	2.76	2.31	3.09	0.23	0.16	8.40
	I 1	2.89	2.695	3.20	0.16	0.11	5.54
	2	2.96	2.44	4.36	0.504	0.34	17.03
	3	2.59	2.21	3.17	0.314	0.21	12.12
	4	2.81	2.54	3.21	0.21	0.14	7.44
	5	2.95	2.53	4.17	0.46	0.31	15.44
	O 1	3.14	2.77	3.78	0.28	0.19	9.17
	2	3.197	2.83	3.78	0.29	0.20	9.09
	3	3.09	2.62	3.84	0.36	0.24	11.62
	4	3.00	2.55	3.38	0.25	0.17	8.47
	M	2.98	2.15	3.46	0.32	0.21	10.59
Liver	N I	46.5	23.2	97.4	11.8	8.0	25.48
	L 1	55.8	46.5	73.0	6.88	4.64	12.33
	2	35.17	26.75	44.7	4.98	3.36	14.15
	3	40.45	31.2	51.1	5.81	3.92	14.35
	4	35.95	26.4	50.4	6.44	4.34	17.89
	5	35.35	27.8	43.4	4.25	2.87	12.02
	D 1	57.1	48.8	64.2	4.61	3.11	8.03
	2	49.1	34.7	61.4	7.99	5.39	16.26
	3	51.1	37.6	64.9	8.16	5.50	15.97
	4	52.2	36.1	76.6	11.82	7.97	22.64
	5	57.8	47.7	75.8	7.98	5.38	13.80
	I 1	46.1	38.5	55.3	4.70	3.17	10.20
	2	51.9	44.6	62.4	5.76	3.89	11.10
	3	37.4	29.9	42.5	4.18	2.82	11.18
	4	43.5	33.3	63.1	8.13	5.49	18.69
	5	46.0	32.7	64.9	11.17	7.53	24.31
	O 1	62.9	40.9	117.0	22.10	14.91	35.14
	2	46.6	33.1	82.6	13.70	9.24	29.40
	3	39.8	30.0	49.7	6.23	4.20	15.65
	4	47.6	31.4	72.5	12.10	8.16	25.27
	M	55.6	33.3	74.8	10.93	7.37	19.71

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Kidneys	N I	6.97	3.45	17.28	1.26	0.85	18.08
	L 1	7.51	6.30	9.42	0.88	0.59	11.72
	2	6.68	4.67	8.21	1.10	0.74	16.47
	3	6.15	5.56	7.34	0.57	0.38	9.24
	4	6.01	5.05	6.90	0.50	0.34	8.37
	5	6.08	4.92	7.59	0.80	0.54	13.13
	D 1	8.02	6.77	10.04	1.22	0.82	15.21
	2	7.37	5.74	9.16	1.10	0.74	14.92
	3	7.17	6.32	8.30	0.51	0.34	7.11
	4	6.62	5.08	8.00	0.84	0.56	12.61
	5	6.58	5.68	7.80	0.57	0.38	8.63
	I 1	7.62	7.11	8.73	0.53	0.36	7.01
	2	7.34	5.60	8.94	0.99	0.67	13.46
	3	6.58	5.06	7.83	0.71	0.48	10.82
	4	6.43	5.48	7.78	0.78	0.53	12.19
	5	6.89	4.68	10.39	1.85	1.25	26.88
	O 1	7.88	6.16	10.24	1.14	0.77	14.44
	2	7.57	6.26	9.75	0.91	0.62	12.03
	3	7.48	5.68	9.99	1.14	0.77	15.24
	4	7.63	6.61	9.07	0.82	0.56	10.78
	M	8.08	6.37	10.44	1.04	0.70	12.86
Spleen	N I	0.531	0.035	1.714	0.214	0.144	40.30
	L 1	0.654	0.433	1.006	0.202	0.136	30.88
	2	0.618	0.294	1.080	0.258	0.174	41.75
	3	0.495	0.210	0.976	0.214	0.144	43.23
	4	0.462	0.197	0.890	0.182	0.123	39.39
	5	0.572	0.048	0.955	0.275	0.186	48.08
	D 1	0.768	0.314	1.334	0.299	0.202	38.95
	2	0.577	0.309	0.805	0.171	0.115	29.63
	3	0.706	0.375	1.353	0.266	0.179	37.67
	4	0.490	0.285	0.826	0.147	0.099	30.00
	5	0.637	0.295	1.239	0.289	0.195	45.36
	I 1	0.795	0.476	1.712	0.363	0.245	45.66
	2	0.669	0.323	1.442	0.293	0.198	43.80
	3	0.517	0.313	0.980	0.186	0.126	35.98
	4	0.504	0.286	0.857	0.148	0.100	29.45
	5	0.644	0.327	1.440	0.314	0.212	48.76
	O 1	0.697	0.509	1.095	0.167	0.113	23.96
	2	0.590	0.293	0.779	0.186	0.126	31.53
	3	0.800	0.295	1.988	0.457	0.308	57.13
	4	0.729	0.346	0.928	0.159	0.107	21.81
	M	0.680	0.270	1.250	0.274	0.185	40.26

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Thymus	N I	1.230	0.334	2.720	0.451	0.304	36.64
	L 1	1.428	0.762	2.380	0.538	0.373	37.67
	2	1.187	0.604	2.120	0.399	0.269	33.61
	3	1.108	0.806	1.730	0.244	0.165	22.02
	4	1.268	0.959	1.540	0.241	0.163	19.01
	5	1.437	0.942	2.330	0.454	0.306	31.59
	D 1	1.415	0.632	1.900	0.360	0.243	25.43
	2	1.220	0.738	1.893	0.368	0.248	30.16
	3	1.142	0.435	1.480	0.337	0.227	29.50
	4	1.024	0.692	1.570	0.270	0.182	26.37
	5	1.116	0.524	1.790	0.391	0.264	35.03
	I 1	1.705	0.818	3.315	0.831	0.561	48.74
	2	1.364	0.602	1.960	0.390	0.263	28.60
	3	1.094	0.684	1.380	0.222	0.150	20.29
	4	1.117	0.315	1.720	0.394	0.266	35.25
	5	0.913	0.640	1.625	0.347	0.234	38.01
	O 1	0.977	0.525	1.930	0.487	0.329	49.85
	2	1.113	0.388	2.407	0.608	0.410	54.63
	3	1.137	0.471	1.722	0.402	0.271	35.36
	4	0.991	0.532	1.765	0.417	0.282	42.08
	M	1.420	0.483	2.370	0.601	0.405	42.29
Testicles	N I	2.61	0.470	4.93	0.729	0.492	27.93
	L 1	2.44	1.780	2.93	0.360	0.240	14.84
	2	2.77	1.950	3.54	0.530	0.360	19.03
	3	2.57	2.070	3.59	0.420	0.280	16.38
	4	2.51	1.880	3.01	0.420	0.280	16.61
	5	2.41	1.890	2.93	0.300	0.200	12.46
	D 1	2.82	1.960	3.53	0.540	0.360	19.14
	2	2.71	1.810	3.73	0.460	0.310	16.78
	3	2.90	2.330	3.81	0.440	0.300	15.27
	4	2.54	1.660	3.27	0.450	0.300	17.51
	5	2.37	1.550	3.10	0.500	0.340	21.01
	I 1	2.76	1.880	3.45	0.510	0.340	18.48
	2	2.72	2.100	3.49	0.470	0.320	17.35
	3	2.85	2.230	3.91	0.510	0.340	17.93
	4	2.63	1.770	3.39	0.460	0.310	17.60
	5	2.43	1.740	3.49	0.530	0.360	21.81
	O 1	3.29	1.160	4.22	0.980	0.660	29.64
	2	2.76	1.940	3.78	0.610	0.414	22.25
	3	2.63	1.160	3.82	0.850	0.570	32.28
	4	2.65	2.050	3.49	0.390	0.260	14.68
	M	2.59	1.810	3.56	0.490	0.330	18.87

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Brain	N I	5.11	3.33	8.16	0.87	0.58	16.92
	L 1	4.59	3.35	5.62	0.67	0.45	14.62
	2	4.82	3.77	6.48	0.92	0.62	19.07
	3	4.37	3.85	4.58	0.21	0.14	4.76
	4	4.26	3.70	5.12	0.42	0.29	9.95
	5	4.49	3.79	5.34	0.41	0.28	9.20
	D 1	5.49	4.30	6.58	0.69	0.47	12.56
	2	4.66	3.84	5.26	0.41	0.28	8.84
	3	4.77	4.06	5.49	0.47	0.31	9.74
	4	4.36	3.58	5.30	0.52	0.35	11.92
	5	4.77	3.91	5.68	0.63	0.43	13.22
	I 1	4.76	3.71	5.52	0.54	0.364	11.34
	2	4.83	4.39	5.44	0.39	0.264	8.10
	3	4.44	3.78	4.93	0.384	0.26	8.65
	4	4.21	3.67	4.81	0.375	0.25	8.91
	5	4.76	3.66	6.29	0.764	0.52	16.06
	O 1	5.49	4.33	6.52	0.70	0.47	12.71
	2	5.16	4.24	7.08	0.84	0.57	16.49
	3	5.18	3.94	6.52	0.94	0.64	18.17
	4	5.25	4.31	5.86	0.50	0.34	9.49
	M	5.41	4.73	6.70	0.51	0.34	9.31
Thyroid	N I	0.12324	0.0504	0.730	0.0757	0.05106	61.47
	L 1	0.09878	0.0661	0.1474	0.0256	0.01727	25.92
	2	0.09074	0.0650	0.1145	0.0172	0.01160	18.96
	3	0.09814	0.0722	0.1253	0.0133	0.00797	13.35
	4	0.12645	0.0620	0.191	0.0353	0.02380	27.92
	5	0.11624	0.0806	0.145	0.02514	0.01696	21.63
	D 1	0.10780	0.0656	0.1318	0.0271	0.0183	25.14
	2	0.11230	0.0720	0.1163	0.0256	0.0173	22.79
	3	0.12380	0.0987	0.1619	0.0201	0.0136	16.26
	4	0.11460	0.0726	0.128	0.0267	0.0181	23.30
	5	0.12680	0.0817	0.3351	0.0706	0.0476	55.68
		(0.10360)	(0.0817)	(0.1239)	(0.0140)	(0.0095)	(13.51)
	I 1	0.12270	0.0907	0.200	0.0316	0.0213	25.71
	2	0.13950	0.0862	0.201	0.0344	0.0232	24.65
	3	0.14800	0.0822	0.218	0.0342	0.0231	23.10
		(0.14027)	(0.0822)	(0.1822)	(0.0313)	(0.0211)	(22.31)
	4	0.18370	0.1010	0.392	0.0957	0.0645	52.07
		(0.1261)	(0.101)	(0.1585)	(0.0248)	(0.0167)	(19.69)
	5	0.14587	0.1108	0.201	0.0257	0.0173	17.68
	O 1	0.13110	0.0569	0.3211	0.0692	0.0467	52.80
		(0.10990)	(0.0569)	(0.1615)	(0.0294)	(0.0198)	(26.74)
	2	0.09960	0.0693	0.149	0.0238	0.0161	23.89

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Thyroid—Continued	O 3	0.10030	0.0745	0.1825	0.0222	0.0150	21.71
	4	0.14950	0.0664	0.3135	0.0739	0.0499	49.51
		(0.13130)	(0.0664)	(0.227)	(0.0525)	(0.0354)	(39.97)
	M	0.16990	0.0615	1.230	0.2457	0.1657	144.65
		(0.11410)	(0.0615)	(0.2092)	(0.0343)	(0.0231)	(30.04)
Parathyroids	N I	0.00692	0.00109	0.02175	0.00278	0.00187	40.13
	L 1	0.00620	0.00353	0.00787	0.00093	0.00063	15.00
	2	0.00500	0.00284	0.00735	0.00115	0.00078	23.00
	3	0.00490	0.00268	0.00769	0.00141	0.00095	28.76
	4	0.00464	0.00203	0.00669	0.00123	0.00083	26.51
	5	0.00530	0.00356	0.00887	0.00151	0.00102	28.40
	D 1	0.00788	0.00431	0.01216	0.00263	0.00177	33.39
	2	0.00544	0.00252	0.00796	0.00154	0.00104	28.30
	3	0.00665	0.00428	0.00955	0.00161	0.00109	24.16
	4	0.00552	0.00296	0.00954	0.00221	0.00149	40.03
	5	0.00698	0.00240	0.01235	0.00341	0.00230	48.85
	I 1	0.00609	0.00482	0.01053	0.00164	0.00110	26.88
	2	0.00658	0.00384	0.01115	0.00210	0.00142	31.92
	3	0.00611	0.00355	0.01140	0.00202	0.00136	33.09
	4	0.00560	0.00251	0.00945	0.00207	0.00140	37.02
	5	0.00951	0.00559	0.01747	0.00351	0.00237	36.96
	O 1	0.00841	0.00426	0.01545	0.00309	0.00208	36.74
	2	0.00647	0.00346	0.01435	0.00279	0.00188	43.12
	3	0.00629	0.00266	0.00994	0.00163	0.00110	25.91
	4	0.00490	0.00178	0.00721	0.00154	0.00104	31.43
	M	0.00654	0.00346	0.01155	0.00215	0.00145	32.89
Hypophysis	N I	0.01540	0.00728	0.0294	0.00310	0.00209	20.27
	L 1	0.01308	0.00820	0.01735	0.00307	0.00207	23.47
	2	0.01434	0.01016	0.01745	0.00264	0.00178	18.42
	3	0.01254	0.01105	0.01445	0.00102	0.00069	8.10
	4	0.01210	0.01034	0.01372	0.00124	0.00084	10.25
	5	0.01156	0.00909	0.01410	0.00219	0.00148	18.95
	D 1	0.01578	0.01325	0.02048	0.00236	0.00159	14.95
	2	0.01401	0.01029	0.01858	0.00217	0.00146	15.48
	3	0.01475	0.01238	0.01856	0.00174	0.00117	11.79
	4	0.01121	0.00792	0.01516	0.00203	0.00137	18.10
	5	0.01385	0.00961	0.01745	0.00244	0.00165	17.61
	I 1	0.01400	0.01096	0.01654	0.00176	0.00119	11.93
	2	0.01595	0.01205	0.02005	0.00253	0.00171	15.87
	3	0.01330	0.01005	0.01770	0.00231	0.00156	17.37
	4	0.01352	0.00960	0.01750	0.00252	0.00170	18.65
	5	0.01460	0.01282	0.01960	0.00235	0.00159	16.10

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Hypophysis—Continued	O 1	0.01665	0.01223	0.02090	0.00256	0.00173	15.38
	2	0.01456	0.01115	0.01826	0.00185	0.00125	12.71
	3	0.01493	0.00994	0.01795	0.00255	0.00172	17.08
	4	0.01505	0.01095	0.02043	0.00278	0.00188	18.47
	M	0.01706	0.01239	0.02135	0.00227	0.00153	13.32
Suprarenals	N I	0.2088	0.0804	0.530	0.07503	0.05061	35.93
	L 1	0.1780	0.070	0.279	0.0526	0.03549	29.55
	2	0.2217	0.115	0.433	0.0846	0.05706	38.11
	3	0.2590	0.145	0.508	0.1187	0.08006	45.83
	4	0.2060	0.154	0.286	0.0403	0.02718	19.56
	5	0.2240	0.138	0.293	0.0496	0.03346	22.14
	D 1	0.1836	0.091	0.299	0.0543	0.0366	29.57
	2	0.2093	0.103	0.269	0.0576	0.0389	27.52
	3	0.1978	0.138	0.340	0.0680	0.0459	34.39
	4	0.2056	0.133	0.271	0.0442	0.0298	21.60
	5	0.2659	0.114	0.582	0.1317	0.0888	49.52
	I 1	0.1980	0.132	0.313	0.0479	0.0323	24.20
	2	0.2240	0.095	0.468	0.1038	0.0700	46.55
	3	0.1860	0.092	0.292	0.0592	0.0399	31.83
	4	0.2210	0.131	0.367	0.0690	0.0468	31.36
	5	0.2640	0.132	0.572	0.1330	0.0899	50.49
	O 1	0.2360	0.136	0.356	0.0644	0.0434	27.29
	2	0.2220	0.133	0.383	0.0836	0.0564	37.66
	3	0.2180	0.127	0.316	0.0549	0.0370	25.18
	4	0.1970	0.080	0.423	0.0983	0.0663	49.89
	M	0.1640	0.088	0.281	0.0575	0.0388	35.08
Pineal	N I	0.00885	0.00273	0.01873	0.00245	0.00165	27.68
	L 1	0.00654	0.00442	0.00899	0.00139	0.00938	21.25
	2	0.00623	0.00352	0.01035	0.00183	0.00123	29.38
	3	0.00643	0.00337	0.00890	0.00179	0.00121	27.84
	4	0.00602	0.00315	0.00834	0.00151	0.00102	25.11
	5	0.00653	0.00440	0.00777	0.00114	0.00077	17.46
	D 1	0.00832	0.00494	0.01415	0.00269	0.00181	32.33
	2	0.00643	0.00447	0.00836	0.00113	0.00076	17.57
	3	0.00829	0.00334	0.01173	0.00232	0.00156	27.92
	4	0.00737	0.00495	0.01236	0.00217	0.00146	29.44
	5	0.00744	0.00495	0.00930	0.00149	0.00101	20.02
	I 1	0.00755	0.00482	0.01004	0.00180	0.00121	23.86
	2	0.00824	0.00625	0.01012	0.00134	0.00090	16.26
	4	0.00678	0.00228	0.00851	0.00163	0.00110	24.04
	4	0.00672	0.00321	0.01045	0.00221	0.00149	32.83
	5	0.00806	0.00489	0.01050	0.00169	0.00114	20.97

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Pineal—Continued	O 1	0.00815	0.00469	0.01362	0.00299	0.00202	36.69
	2	0.00751	0.00588	0.01077	0.00163	0.00110	21.69
	3	0.00694	0.00263	0.01005	0.00228	0.00154	32.85
	4	0.00767	0.00456	0.01202	0.00265	0.00179	34.55
	M	0.00820	0.00307	0.01150	0.00212	0.00143	25.85
Popliteal lymph nodes	N I	0.13573	0.0525	0.382	0.04873	0.03287	35.90
	L 1	0.1320	0.096	0.193	0.0321	0.02165	24.32
	2	0.1200	0.084	0.201	0.0317	0.02139	26.42
	3	0.1230	0.082	0.163	0.0247	0.01666	20.08
	4	0.1110	0.074	0.177	0.0316	0.02131	28.47
	5	0.1100	0.068	0.138	0.0221	0.01491	20.09
	D 1	0.1583	0.123	0.228	0.0340	0.0229	21.48
	2	0.1471	0.117	0.201	0.0266	0.0179	18.08
	3	0.1149	0.056	0.146	0.0278	0.0188	24.18
	4	0.1187	0.077	0.149	0.0224	0.0151	18.86
	5	0.0988	0.061	0.149	0.0302	0.0203	30.54
	I 1	0.1570	0.105	0.208	0.0318	0.0225	20.25
	2	0.1310	0.085	0.196	0.0335	0.0226	25.57
	3	0.1190	0.079	0.192	0.0319	0.0215	26.81
	4	0.1160	0.078	0.147	0.0238	0.0161	20.53
	5	0.0950	0.052	0.177	0.0342	0.0231	36.19
	O 1	0.1060	0.075	0.153	0.0297	0.0200	28.02
	2	0.1370	0.072	0.196	0.0430	0.0296	31.31
	3	0.1530	0.085	0.231	0.0385	0.0260	25.16
	4	0.1380	0.078	0.248	0.0488	0.0329	35.36
	M	0.1490	0.077	0.283	0.0629	0.0424	42.20
Axillary lymph nodes	N I	0.09722	0.01865	0.2425	0.03779	0.02549	38.87
	L 1	0.084	0.059	0.125	0.02245	0.01514	26.72
	2	0.075	0.037	0.172	0.0397	0.02678	52.93
	3	0.078	0.054	0.142	0.0263	0.01774	33.72
	4	0.068	0.051	0.097	0.0146	0.00985	21.47
	5	0.065	0.041	0.088	0.0136	0.00917	20.92
	D 1	0.1089	0.078	0.188	0.0308	0.0208	28.28
	2	0.0817	0.035	0.120	0.0238	0.0161	29.14
	3	0.0848	0.037	0.111	0.0229	0.0155	26.99
	4	0.0716	0.036	0.098	0.0195	0.0132	27.21
	5	0.0652	0.044	0.077	0.0110	0.0074	16.88
	I 1	0.1010	0.050	0.140	0.0280	0.0189	27.75
	2	0.083	0.058	0.106	0.0166	0.0102	20.00
	3	0.083	0.050	0.114	0.0231	0.0156	27.83
	4	0.076	0.034	0.105	0.0222	0.0150	29.16
	5	0.063	0.041	0.091	0.0142	0.0096	22.57

TABLE II—*Continued.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Axillary lymph nodes—Continued	O 1	0.072	0.050	0.088	0.0132	0.0089	18.33
	2	0.074	0.039	0.103	0.0180	0.0120	24.37
	3	0.112	0.050	0.237	0.0480	0.0330	43.04
	4	0.097	0.063	0.160	0.0290	0.0194	29.69
	M	0.102	0.036	0.240	0.0520	0.0350	51.02
Deep cervical lymph nodes	N I	0.08207	0.02052	0.211	0.03712	0.02504	45.23
	L 1	0.067	0.031	0.102	0.01830	0.01234	27.34
	2	0.066	0.033	0.099	0.02356	0.01589	35.70
	3	0.071	0.039	0.110	0.0231	0.01558	32.54
	4	0.051	0.033	0.087	0.0155	0.01046	30.39
	5	0.111	0.046	0.313	0.0879	0.05929	79.19
		(0.069)	(0.046)	(0.111)	(0.0214)	(0.0144)	(30.92)
	D 1	0.0957	0.062	0.147	0.0331	0.0223	34.56
	2	0.0926	0.038	0.182	0.0378	0.0255	40.81
	3	0.0758	0.046	0.199	0.0431	0.0291	56.83
	4	0.0568	0.022	0.138	0.0311	0.0210	54.75
	5	0.0758	0.044	0.236	0.0540	0.0365	71.28
		(0.0580)	(0.044)	(0.073)	(0.0090)	(0.0061)	(15.52)
	I 1	0.095	0.059	0.221	0.0452	0.0305	47.58
	2	0.078	0.051	0.096	0.0156	0.0105	20.00
	3	0.065	0.042	0.094	0.0155	0.0105	23.85
	4	0.068	0.046	0.136	0.0244	0.0165	35.86
		(0.061)	(0.046)	(0.0766)	(0.0098)	(0.0066)	(16.07)
	5	0.108	0.053	0.278	0.0656	0.0436	59.81
		(0.089)	(0.053)	(0.1681)	(0.0327)	(0.0220)	(36.66)
	O 1	0.085	0.059	0.132	0.0240	0.01620	28.26
	2	0.082	0.047	0.126	0.0214	0.0150	26.10
	3	0.093	0.034	0.143	0.0360	0.0240	42.90
	4	0.0904	0.046	0.227	0.0310	0.0210	34.46
	M	0.0902	0.032	0.311	0.0610	0.0410	67.65
		(0.0724)	(0.032)	(0.1449)	(0.0239)	(0.0161)	(32.93)
Mesenteric lymph nodes	N I	1.828	0.72	3.71	0.642	0.433	35.12
	L 1	1.543	0.868	2.068	0.352	0.237	22.81
	2	1.428	0.518	1.981	0.442	0.298	30.95
	3	1.530	0.955	2.560	0.421	0.284	27.52
	4	1.284	1.080	2.000	0.268	0.181	20.87
	5	1.347	0.810	2.280	0.389	0.262	28.88
	D 1	1.847	1.248	2.340	0.344	0.232	18.62
	2	1.468	0.886	1.974	0.313	0.211	21.32
	3	1.470	0.960	2.090	0.312	0.210	21.22
	4	1.292	1.066	1.650	0.163	0.110	12.61
	5	1.269	0.870	1.650	0.249	0.168	19.62

TABLE II—*Concluded.*

Organ	Group	Arithmetical mean	Minimum	Maximum	Standard deviation	Probable error	Coefficient of variation
		gm.	*gm.*	*gm.*	± *gm.*	± *gm.*	*per cent*
Mesenteric lymph nodes—Continued	I 1	1.628	0.902	2.492	0.434	0.293	26.66
	2	1.549	0.883	1.890	0.302	0.204	19.43
	3	1.534	1.269	2.030	0.225	0.152	14.67
	4	1.417	0.980	2.430	0.392	0.264	27.67
	5	1.446	0.940	2.100	0.324	0.218	22.41
	O 1	2.376	1.105	3.430	0.857	0.578	27.65
	2	3.067	1.185	6.910	1.488	1.004	48.52
	3	2.340	1.640	3.270	0.539	0.364	23.03
	4	2.670	2.110	3.440	0.393	0.265	14.72
	M	2.220	1.450	2.880	0.389	0.262	17.51

EFFECTS OF LIGHT ON NORMAL RABBITS, WITH ESPECIAL REFERENCE TO THE ORGANIC REACTION.

III. Analysis of Organ Weights.

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In Paper I of this series the purpose of the investigation was stated, and the experimental methods, the general clinical observations, and postmortem findings were described (1). The results of the experiment, in the form of organ weights, were presented in Paper II (2). In the present paper these results are discussed.

DISCUSSION.

It is evident that in considering the relationship between environment and the state of the animal organism, many factors are concerned, the effects and relative importance of which are difficult and perhaps impossible to estimate accurately. From a broad point of view, however, the state of the organism at any one time may be said to be conditioned by all the operative forces of physical environment together with the organism's response to their action. While, in the rabbit, variations in organ weight as well as more subtle functional changes, such as the body's reaction to certain disease processes, appear to be influenced by the amount and degree of change in the hours of sunshine (1), these cannot be the only environmental influences implicated. Other characteristics of sunlight and a number of totally different forces, some perhaps unsuspected as yet, undoubtedly participate in producing the alterations. In our experiment, the influence of light has been studied only under the following conditions: (1) constant illumination, (2) absence of shorter ultra-violet rays, and (3) continued exclusion of light. Although our present attention in this experiment is focused upon the alterations in organ weight asso-

ciated with these particular variations of light environment, it should be emphasized that other factors which might exercise an effect upon the mass relationships of organs will have to be considered before the full influence of the light factor can finally be estimated.

The evaluation of the results depends first, upon the significance to be attached to variations in weight of a given order with due regard for the occurrence of spontaneous variations of a similar order, and second, on the nature of the apparent effect produced. As has been mentioned, the experimental conditions employed involve but a few of the hypothetical factors that might affect organ weights. It was not possible to control all these, but an effort was made to equalize the influence of a number of conditions so that any effect they might have would be common to both the experimental animals and controls. The rabbits, of comparable breeds and age, were kept assembled for several weeks before the experiment in order to insure uniform periods of caging under the same environmental conditions. In addition, the same system of feeding was employed throughout the entire period of observation. Furthermore, a regular routine with reference to the time and order of postmortem examinations was followed, the experimental groups being killed on a certain day and the controls on the following day. The number of rabbits comprising each subgroup—10—was selected as being small enough to insure a satisfactory postmortem examination, including the accurate weighing of organs and, at the same time, large enough to furnish data which could be used statistically. Finally, the comparatively short duration of the experiment tended to minimize the wide fluctuations of organ weights which occur in material examined over comparatively long intervals of time (3).

An idea of the uniformity of the various groups is obtained from the coefficients of variation (2). On the whole, the animal groups exposed to constant illumination or kept in the dark had the most uniform organ weights in the case of most of the organs, and next came the groups kept in an ordinary room (inside controls), while the rabbits which had recently been brought to the laboratory (outside controls) were relatively irregular in this respect. In the case of most organs, the coefficients of variation of the experimental groups are much smaller than those of the 350 normal rabbits previously studied (3), a fact which could hardly be accounted for upon the basis of the differ-

ences in the numbers of animals. However, the rabbits of the experimental series were a far more homogeneous animal stock than the large normal group.

From a statistical point of view, the significance to be attached to variations of organ weight of a given order is directly dependent upon the magnitude of the probable errors of these values. The probable errors in the case of our experiment were large and it would appear that little statistical significance could be attributed to the variations observed. However, in an analysis of the organ weights of two groups of normal rabbits, comprising 350 and 295 animals respectively, the probable errors of the mean weights of all the organs were extremely large, yet the average or mean weights of each organ were almost exactly the same in both groups (4). Similar conditions have been found to obtain with several groups of 10 normal rabbits killed on the same days and at a season of the year in which variation in organ weight was known to be marked. This closeness of agreement between mean organ weights would not be expected upon the basis of the magnitude of the probable errors, as shown by the following examples:

Organ		Mean weight	Probable error	Deviation / P. E.
		gm.	± *gm.*	
Liver	A	91.6	8.50	
Actual	B	88.2	11.33	0.24
Heart	A	5.02	0.78	
Actual	B	5.11	0.57	0.0092
Parathyroids	A	0.00653	0.00125	
Relative	B	0.00655	0.00161	0.04
Suprarenals	A	0.164	0.0376	
Relative	B	0.164	0.0400	0.0

These observations are highly significant and suggest that with material of this kind, the usual method of statistical evaluation based upon the ratio of the difference of mean weights to its probable error may not always be entirely satisfactory, although, as should be pointed out, additional data will have to be available before any general deduc-

tions on this point can be drawn. For the present, however, it seems advisable not to place too much stress on the results of statistical comparisons, or, in makng such comparisons, to bear in mind the fact that, on the basis of comparisons which have been made, the differences found between the weights of comparable groups of normal animals are as a rule about 1/3 to 1/2 of the difference that might be expected. If this fact is taken into consideration in analyzing the results the differences in weight that are found are, in many instances, of considerable statistical significance.

However, if the usual basis of evaluation be followed, little statistical significance can be attached, in most instances, to differences in mean organ weight if a group to group comparison is made, but between the highest and lowest group values in a given series the difference is frequently sufficiently large to make it of probable significance. The controls resemble the two experimental series in these respects. With some organs, the ratios of the differences between extreme mean weights to the probable errors are larger with control than with experimental groups as might be expected in view of the spontaneous variations in organ weights of normal rabbits and, as will be seen, became of the general tendency toward organ weight uniformity shown by the groups exposed to constant light or to darkness. Furthermore, the differences that are found in comparing the mean organ weights of experimental groups with their appropriate controls or with so called normal values, as well as the differences found between experimental groups, vary from those of little or no statistical significance to those that have some statistical significance. In the majority of cases, however, the results of the statistical analysis have chiefly a negative value and the decision as to the significance of the findings has to be made on some other basis, such as the trend of a given curve or the direction of a possible change in weight of an experimental group of animals as compared with controls or with another experimental group. The application of this method of evaluation will become apparent as the results recorded in this investigation are discussed.

The present analysis of organ weights is primarily based upon a comparison with the mean organ weights from a group of 350 normal rabbits (3) because of the fact that the organ weights of the control

animals are not constant values but constitute a moving base. By the use of curves which have been plotted upon a basis of percentage deviation from these so called mean normal values, the general trend of organ weight obtaining in the control rabbits during the experiment is easily seen and the variations of the experimental groups are more readily appreciated. Only the curves for relative weights of organs have been used in the present discussion, except in the case of the gastrointestinal mass.

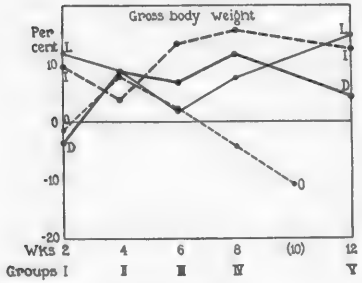
The organs have been considered in the following order: gross and net body weight, the gastrointestinal mass, the heart, the kidneys, the liver, the brain, the glands of internal secretion, the testicles, the spleen and thymus, and representative lymph nodes.

The terms, Groups I to V, used in this discussion refer to the groups of rabbits killed and examined 2, 4, 6, 8, and 12 weeks from the beginning of the experiment, these periods being the duration of exposure to conditions of constant illumination or of constant darkness. There is no Group V of outside controls but a series of normal rabbits, designated March normals, examined 10 weeks after the beginning of the experiment, is included.

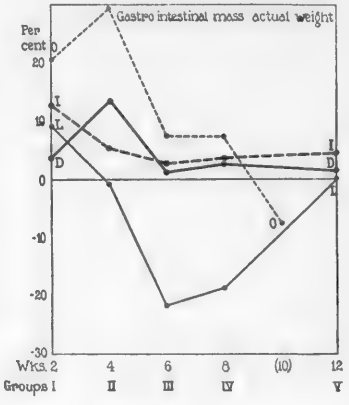
Gross and Net Body Weights.—The curves of gross and net body weights (Text-figs. 1 and 2) corresponding to the four animal series—light and dark groups, inside and outside controls—are identical in form, but the relative positions of the curves with respect to the mean value for the 350 normal rabbits are more accentuated in the case of the net body weights. All the curves of both gross and net body weights show irregularities, but those representing the successive groups of rabbits exposed to constant light are fairly regular and, in general, on an increasingly high level, well above the mean normal. The same is generally true of the curves for the inside controls. On the other hand, the curves for the dark room groups begin below the base line, and while they subsequently rise above it, their general level is below those of the light series and inside controls. The curves representing the weights of the outside control animals are generally below the others. Their low positions, corresponding to Group IV and the March normals, are influenced by the fact that at this time of year the available rabbit stock consists almost entirely of comparatively young animals.

Gastrointestinal Mass.—The curves illustrating variations in the actual weight of the gastrointestinal mass (including stomach, intestine, mesentery, omentum, and gastrointestinal contents) show definite differences in the case of the rabbits in the light environment (Text-fig. 3). The curve of the inside control groups is quite uniform and only slightly higher than the base line. With the exception of one irregularity (Group II) the same is true of the curve of the animals exposed to constant darkness. On the other hand, the curve of the light groups shows two

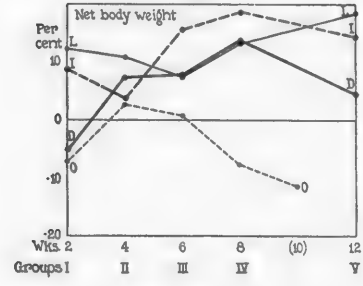
pronounced fluctuations, and its general level, moreover, is lower than those for the inside controls and for the dark series, while with Groups III and IV its position is considerably below the mean normal. The curve for the outside controls is the highest of the four; its general direction is downward, however, to a level approximating the mean normal. It will subsequently be seen that, in respect to the trend of weight variations, the gastrointestinal mass behaves in much the same



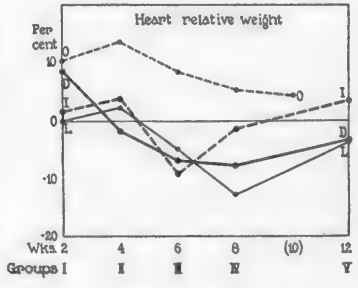
TEXT-FIG. 1.



TEXT-FIG. 3.



TEXT-FIG. 2.



TEXT-FIG. 4.

The curves in these and all other text-figures show the variations in mean weight as percentage deviation from the mean weights of 350 normal rabbits (3). The number of weeks refers, in each instance, to the time the groups of rabbits had been under conditions of constant light or of darkness, *i.e.* 2 weeks = Group I, 3 weeks = Group II, etc. The following system of representation is used in all text-figures:

Light groups ———— Inside controls - - - - - -
Dark groups ———— Outside controls - - - - -

manner as the parenchymatous organs. This is particularly noticeable in the case of the light room animals.

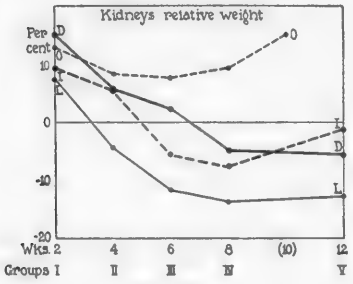
Heart.—In the case of the heart, the curves illustrating the relative weights obtained from the dark and light series are, in general, on a slightly lower level than that for the inside controls (Text-fig. 4). In the first part of the experiment (Groups I, II, and III), all three curves are falling, but with Groups IV and V, the inside control curve rises fairly abruptly to above the mean normal, while the curves for both experimental groups do not rise until Group V and then do not reach this level. The curve for the outside controls is very uniform and well above the others.

Kidneys.—The mean relative weights of the kidneys in rabbits under conditions of constant illumination progressively diminish in a remarkably uniform manner from a value slightly above the mean normal to one well below it. As is shown in Text-fig. 5, the curve for these groups is very regular and occupies the lowest position of the four. The curves for the dark and inside control groups also show a progressive but less pronounced and less regular decline from initial values somewhat above the mean normal, and there is a slight tendency for the values of the dark series to be larger than those of the inside controls. The curve for the outside controls is generally above the others, as it was in the case of the heart.

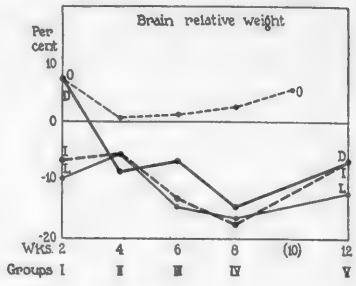
Liver.—The changes in the relative weights of the liver in association with conditions of constant light and of constant darkness were striking. During the time of this experiment, the relative liver weights of the inside control rabbits were in fair approximation to the mean normal but with a tendency toward smaller values (Text-fig. 6). The curve for the outside controls shows wider fluctuations, but there appeared to be a general tendency for the relative weights to be somewhat above the mean normal, although the value of Group III was considerably smaller than the others. In the case of the rabbits living under conditions of constant darkness, the weights of all groups were large and, on the whole, considerably above the mean normal. The curve begins at a high level and after one fall (Groups II) rises progressively to a point with Group V, which is approximately the same as that of Group I. On the other hand, the relative liver weights of the animals in an environment of constant light were, with the exception of Group I, extremely small. In both the first light and dark groups exposed to these conditions for 2 weeks, the relative weights of the liver were large and much larger than for the inside controls. With longer periods of exposure, however, the general tendency of relative liver weights was toward small values for the light and toward large values for the dark groups, respectively.

Brain.—In the case of the brain, the curves show that the relative weights of all the light and inside control groups were considerably below the mean normal, and with the exception of the first group, the same was true for the dark animals (Text-fig. 7). On the other hand, the relative weights of the outside con-

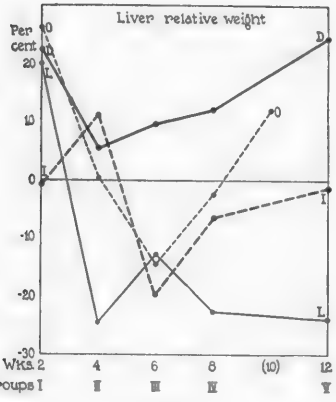
trols were all slightly higher than this value. The curve of the light series is, in general, the lowest, and that of the dark groups the highest, of the three representing animals caged within doors. The dark room curve is the most irregular and its fluctuations are the widest, while the light room curve is slightly but definitely the most regular. It is of interest to note that, on the whole, the variations in relative brain weight, shown by the light, the dark, and the inside control groups, were more marked than those manifested by the heart.



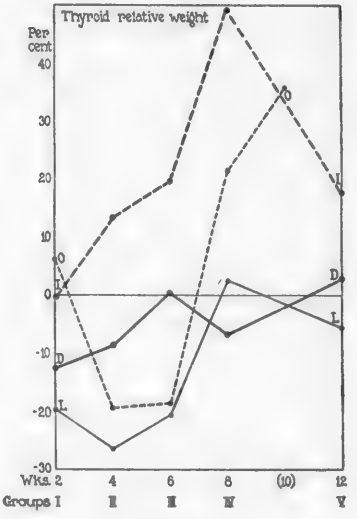
TEXT-FIG. 5.



TEXT-FIG. 7.



TEXT-FIG. 6.

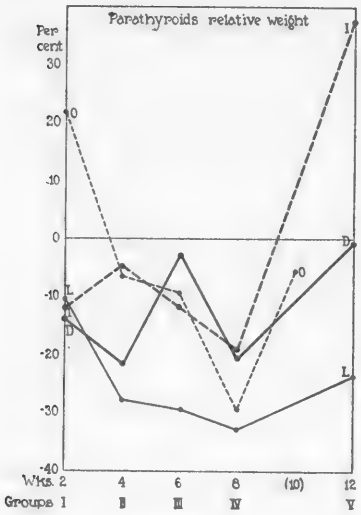


TEXT-FIG. 8.

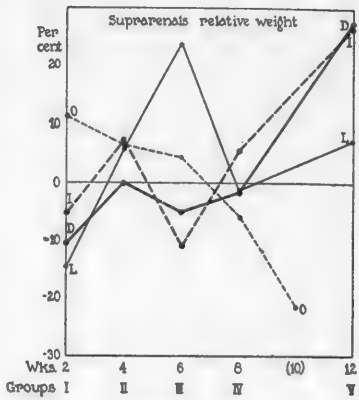
Thyroid.—The curve of relative thyroid weight for the inside control groups (Text-fig. 8) rises abruptly from a point which practically coincides with the mean normal value to a high level with Group IV, and then falls with Group V but to a value equal to that of Group III and higher than those of Groups I and II. There was a similar tendency among rabbits recently removed from outdoor conditions, although in two of the five observations (Groups II and III) the relative weights were very small. The curves for both light and dark groups differ in several respects from the control curves. Both start at lower levels, considerably below the mean normal, and in only three observations (Light IV, Dark III and V) do they reach this value. The general direction of the curve for the dark series is upward, and with the single fluctuation caused by Group III, is remarkably regular; its slope, however, is very gradual, and its final position is just above the mean normal. With the exception of Group IV, the curve for the light series is the lowest of the four. Its first portion (Groups I, II, and III) is far below the mean normal, and its rise with Groups IV and V, carries it only to this value. Although the curve for the rabbits in the constant light environment is not as uniform as that of the groups kept in constant darkness, the magnitude of its single fluctuation is much less pronounced than those of the inside and outside control curves, which swing first in one direction and then in another.

It would appear from these observations, that at a time when the trend of average relative weights of the thyroid of rabbits caged for some weeks in an ordinary animal room, or recently brought into the laboratory, was toward increasingly high levels, much above the mean normal, the general effects of caging for 2 to 12 weeks in environments of constant light or of constant darkness were, first, to restrain or depress this tendency to a marked degree, and, second, toward a stabilization of gland weight at a level below or approaching the mean normal value. In this connection it is interesting to note the group incidence of extremely large thyroids. Thus, there was one such gland in outside Groups I and IV and the March normals, and two in Group III and three in Group IV of the inside controls, while there was none in any of the light groups and only one among the dark room animals in Group V.

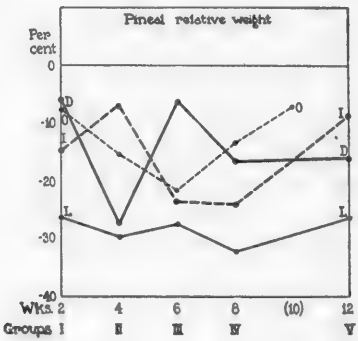
Parathyroids.—In regard to the parathyroid glands, constant illumination was again associated with diminished relative weights, as is clearly brought out in the curves (Text-fig. 9). During the first part of the experiment, the relative weights of the parathyroids of rabbits kept under unaltered conditions for many weeks (Inside Control Groups I, II, III, and IV) were small—from 5 to 15 per cent smaller than the mean normal—while the value of Group V was unusually high —37.5 per cent larger than the mean normal. The coefficients of variation of Groups IV and V were of the same order of magnitude, while that of Groups I, II, and III were slightly smaller (2). The curve for the outside controls begins considerably above the mean normal and ends just below it, but with the exception of its high initial position, its form is like that for the inside controls, while its level, corresponding to Groups II, III, and IV, is also in general agreement. The curve for the dark series is fairly comparable to that for the inside controls except



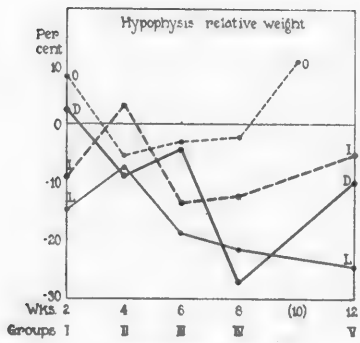
TEXT-FIG. 9.



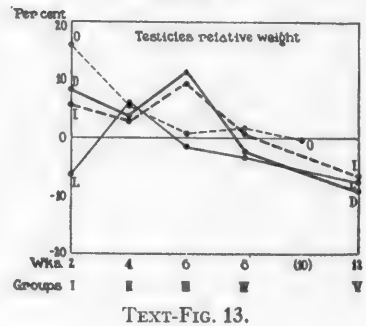
TEXT-FIG. 11.



TEXT-FIG. 12.



TEXT-FIG. 10.



TEXT-FIG. 13.

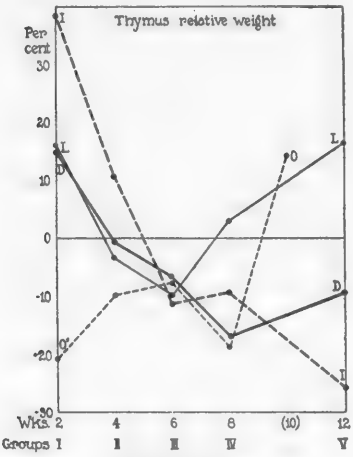
that the final rise corresponding to Group V is less marked. The differences in direction of the two curves, from Groups I to II and from Groups II to III may or may not be significant. The curve illustrating the light series is remarkably regular; it begins at the same general low level as those for the inside controls and dark groups and then falls continuously with Groups II, III, and IV to a much lower level which is only slightly increased with Group V. The terminations of the curves (Groups V) for the inside and outside controls and for the animals in the dark room are all considerably higher than the preceding observations (Groups IV).

Hypophysis.—With the hypophysis, the relative organ weights for the inside controls were, in general, smaller than the mean normal, while those for the outside controls were in fairly close approximation to it, as is shown by the curves in Text-fig. 10. The curve for the dark groups begins slightly above the mean normal (Group I), but with subsequent observations it falls considerably below this value and its general position is well below that for the inside controls. The curve illustrating the relative weights of the light series contrasts sharply with these curves both in respect to position and fluctuations. It occupies the lowest position and is by far the most regular, indicating again both a depressive and a stabilizing effect associated with the environment of constant light.

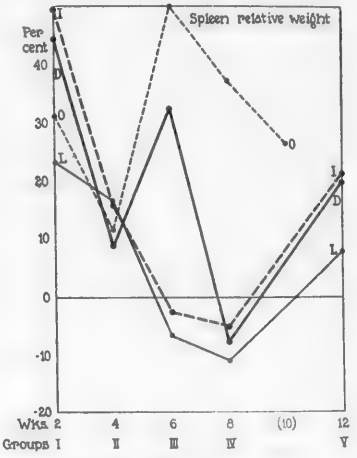
Suprarenals.—Variations in weight of the suprarenal glands of rabbits exposed to constant light or to constant darkness were less apparent than those of many other organs. In the case of recently acquired rabbits, the relative suprarenal weight (Text-fig. 11) steadily diminished from a value slightly above the normal mean as represented by Outside Control Group I to a value considerably below it (March normals). The first three observations on the inside controls showed slight fluctuations about the mean normal, but with Groups IV and V there was a well marked increase in relative weight and the curve rises sharply. Caging under conditions of constant darkness did not appear to have any particular effect upon relative suprarenal weights as compared with those of the inside controls, and except for a tendency toward uniformity of weight in the first four observations, the curve for these groups is quite similar to that for the inside controls. The results obtained from rabbits exposed to constant illumination are irregular and difficult of interpretation. The first two observations are similar to those of the inside controls and the dark series, but with Group III the curve rises sharply to a high point, then falls as abruptly with Group IV to the mean normal, and remains practically at this level with Group V. At the time of these last two observations, the relative weights of the suprarenals in the inside control groups were progressively increasing. The value of Light Group I is the lowest of the four as in the case of the majority of organs. It is possible that observations made earlier than the 2 week period would have shown still lower values and that the increasingly larger weights of Groups II and III represent conditions of readjustment with a subsequent stabilization at a level approximating the mean normal. On the other hand, one might assume, upon the basis of the very large coefficient of variation (2) that the high mean value for Group III was not fairly representative of the

weight of the suprarenals at this time and that if a larger number of rabbits had been examined the average value might have been considerably lower, in which case the trend of relative suprarenal weights for the light series would have been toward a stabilization about the mean normal.

Pineal.—The outstanding feature of the observations on the pineal gland was the consistently low and very uniform relative weights of rabbits exposed to constant illumination. During the time of the experiment, the relative weights of both outside and inside controls (Text-fig. 12) were all lower than the mean normal and, in addition, were subject to rather wide fluctuations. The curve for the dark series shows equally pronounced weight variations in its first part but a tendency toward greater uniformity in its latter half (Groups IV and V). While the direction of this curve is not always the same as that for the inside controls, there is no



TEXT-FIG. 14.



TEXT-FIG. 15.

maintained trend which could be attributed with certainty to the effect of constant darkness. On the other hand, the relative weights of the pineal in the light groups were very small and uniformly so. It is remarkable that with an organ like the pineal, in which errors incident to removal and weighing must occur, the relative weights should be so consistently maintained in the successive light room groups that the curve is almost a straight line. Both the depressive and stabilizing effects on organ weight associated with the constant light environment of this experiment are well exemplified by the pineal gland.

Testicles.—In the case of the testicles (Text-fig. 13), there was practically no difference in the relative weights of the groups kept in the dark room and the inside controls, but there was a slight tendency toward smaller values on the part of the light series.

Thymic Mass.—The thymic mass apparently responded to the influence of constant illumination in a manner somewhat different from the organs already considered. As is seen from the curves (Text-fig. 14), the mean relative weights of the mass in the inside control groups progressively decreased from a high value of 40 per cent above the mean normal (Group I) to 26 per cent below it (Group V). During the same time, the observations for the four groups of outside controls were from 8 to 20 per cent below, while that of the March normals was 14 per cent above, the mean normal. This variation of the latter group from the general low level of the outside controls may, in part, be accounted for by the younger age of the rabbits and perhaps also by the fact that the majority of them had been in the laboratory for 4 weeks. It will be recalled that the inside control groups as well as those placed in the light and dark rooms had been assembled 4 to 6 weeks before the experiment was begun (1). It is possible that, other things being equal, conditions of indoor life are at first associated with a large relative weight of the thymic mass. The curve of relative thymus weights for the dark series is similar to that of the inside controls except that it begins at a lower level and that it rises slightly at the end. While such minor differences may not be of any significance, they suggest a tendency toward stabilization of relative organ weight not observed in the inside control rabbits. In regard to the rabbits exposed to constant illumination, the first part of the curve, corresponding to the first three groups, is almost identical with the curve for the dark room animals. From this point on, however, the curve rises abruptly with Group IV, and continues to rise with Group V to a value practically identical with that of Group I, that is, 17 per cent above the mean normal. At this time, the relative thymus weights of the inside controls were progressively diminishing and the value for Inside Group V was 26 per cent below the mean normal.

In connection with these results, the question arises as to whether well marked differences in relative weights of the thymic mass might not be accounted for on the basis of variations in fat content rather than in actual thymic tissue. However, in the case of Groups V of the light room rabbits and the inside controls, there was little difference in gross and net body weights or in the general state of body fat at autopsy, so that an increase in the weight of the thymic mass due to an accumulation of fat might be as readily assumed in one group as in the other.

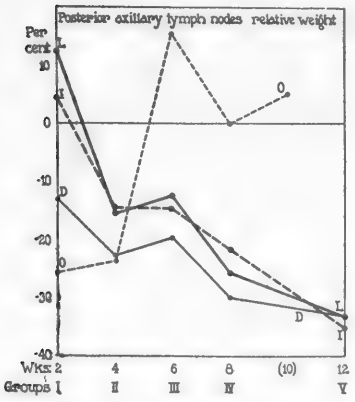
Spleen.—While the results obtained with the spleen do not indicate any striking weight variations which can be associated with the experimental light and dark conditions, the observations of the light groups suggest a slight effect. At the beginning of the experiment, the mean relative weight of the spleen was large in all groups (Text-fig. 15), but that of the light group was much the smallest of the four. From the high initial value, there was a progressive decrease in relative weights of the inside control groups (Nos. III, IV, and V), reaching a value slightly below the mean normal but with Group V somewhat exceeding it. The curve for the light series is similar in form to that of the inside controls, but its general position is somewhat lower and it is more regular. With the dark groups there was a similar general trend toward smaller relative weights. The curve is very like that

for the inside controls with the exception of one pronounced upward fluctuation corresponding to Group III. In the case of the outside control groups the relative weights of the spleen were large, but the shape of the curve suggests a tendency toward decreasing weights which was interrupted by the high value of Group III. There was a marked irregularity in the weight of the spleen in the great majority of the groups as shown by the magnitude of the coefficients of variation (2), and in this respect these observations are in general accord with the two large series of normal rabbits (4). The high mean weights of Groups III of the dark and outside control rabbits were out of line with the prevailing tendency of decreasing weights, but the proportion of heavy organs in both groups indicates that their increased values were fairly representative although their coefficients of variation were of a greater magnitude than those of the preceding and succeeding groups. In this connection it should be mentioned that no pathological processes were found in the rabbits of either group which were considered sufficient to account for the larger size of the spleens. As far as Dark Group III is concerned, all that can be said is that it did not share in the general tendency toward decreased weight shown by other groups of the dark series or by the light and inside control groups during this period.

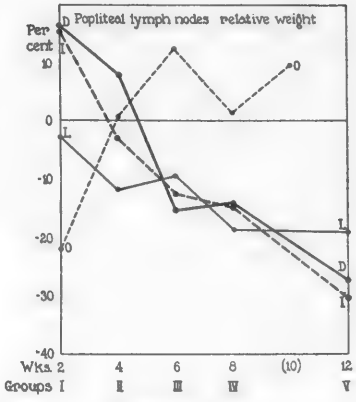
Lymph Nodes.—The last organs to be considered are representative lymph nodes: the popliteal, the posterior axillary, and the deep cervical nodes of both sides, and the main mesenteric mass of lymph nodes. Since similar changes in relative weights occurred in the two groups of superficial nodes, it seems sufficient to discuss but one of them. As will be seen in the curves of the posterior axillary nodes (Text-fig. 16), the relative weights of those of the inside controls decrease progressively from a point (Group I) close to the mean normal to one far below it (Group V). The curve for the outside controls begins (Groups I and II) well below the mean normal but rises with Groups III, IV, and the March normals to a general level slightly above this value. The curve for the dark series is very like that of the inside controls both in direction and position. The relative mean weights of the light groups, on the other hand, are all smaller than those of the dark room rabbits and the inside controls except for the last group the figure for which practically coincides with the final one for the others. In addition, the curve for the rabbits exposed to constant illumination is the most regular.

The trend of the weights of the deep cervical lymph nodes (Text-fig. 18) is in general conformity with that of the posterior axillary and popliteal nodes in the case of all four series of rabbits except for the increased values of Groups V of the inside controls and of the light and dark groups. There were a number of instances in which enlarged nodes were associated with a purulent inflammation of the nasal passages or cranial sinuses and others in which such a condition may have previously existed. In plotting the curves for the deep cervical nodes the weights of a few extremely large nodes in rabbits which showed an extensive purulent exudate in the cranial sinuses or nasal passages have been omitted from Groups V of the light, the dark, and the inside control series.

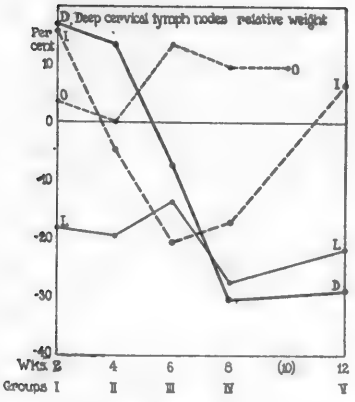
The results obtained with the main mass of mesenteric lymph nodes, as illus-



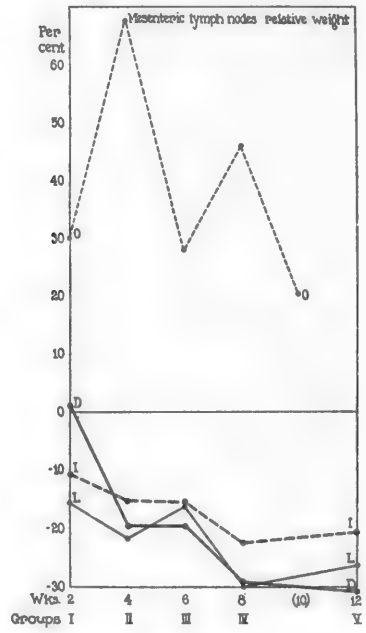
TEXT-FIG. 16.



TEXT-FIG. 17.



TEXT-FIG. 18.



TEXT-FIG. 19.

trated in the curves (Text-fig. 19), show that the relative weights in the case of the animal groups recently brought to the laboratory were much greater than the mean normal and, in addition, were quite irregular, as the marked fluctuations of the curve illustrate. On the other hand, the relative mean weights for the inside controls were somewhat smaller than the mean normal with a slight tendency toward decreasing values; their comparative regularity is shown by the smoothness of the curve. The curves for both experimental groups closely resemble that for the inside controls. With the exception of the first dark group, both curves are slightly but consistently lower, but the difference is a minor one and little significance can be attached to it.

These results may be summarized as follows: With an environment of constant illumination two principal effects upon organ weight were observed. There was a definite tendency toward small relative weights which was particularly striking in the case of the liver, the kidneys, the gastrointestinal mass, the thyroid, the parathyroids, the hypophysis, the pineal, the spleen, and representative lymph nodes; it was less pronounced with the heart, the brain, and the testicles. Moreover, with some organs such as the liver, the thyroid, and the parathyroids, this tendency could be noted when the organs of rabbits living under ordinary indoor conditions were large or were increasing in weight. The thymus appeared to be an exception to this general effect of diminished organ weights, for the last two observations showed increasingly large values, while those of the controls continued to be progressively smaller.

The second effect associated with conditions of constant illumination may be described as a stabilization of organ weight in contrast with the more pronounced irregularities or fluctuations observed in the control groups. The curves illustrating the mean relative organ weights of the groups exposed to the light environment are in most instances more regular than those of the controls, while the smaller coefficients of variation in the majority of the light room organs indicate the comparative weight uniformity within the groups (1).

The effects upon organ weights were less evident in the case of rabbits living under conditions of light exclusion for the same periods of 2 to 12 weeks. A tendency toward smaller mean weights as compared with the control values was observed in the case of the relative weights of the heart, the parathyroids, the hypophysis, the pineal, and the thymus, and to a considerably greater degree with the thyroid. A

tendency toward uniformity or stabilization of organ weight was also observed with some organs, especially the thyroid and the heart. But the most striking feature of the results obtained in this series of animals was the marked and sustained high weight values for the liver. Both the actual and relative weights were very large and were considerably greater than those of the controls. This effect upon the liver was not shared by the other parenchymatous organs, and while no reason for its occurrence can be stated with certainty, it may possibly be associated with an adjustment of metabolic activity. Mention was made in Paper I (1) that some physical deterioration in the rabbits kept in constant darkness was suggested by the body weight curves and the general state of body fat of certain animals at autopsy. The large size of the liver in the groups of dark room rabbits may be significant in this connection.

The lymph nodes as a class showed practically no weight alterations in the rabbits caged under conditions of constant darkness, and only slightly smaller values in the animals kept under constant illumination, other than those which also occurred in the control rabbits living in an unaltered animal room.

The curves illustrating the variations in mean organ weight are of three principal types which, in conjunction with the experimental conditions and the variability of organ weight known to occur spontaneously, are strongly suggestive of a causal relationship between the external factors of physical environment and the mass relationships of organs. The most common type of curve has an initial downward movement with a final upward swing, as is illustrated by the graphs of the gastrointestinal mass, the thymus, and the parathyroids of the animal groups exposed to constant illumination and those of the heart and the liver of the rabbits living in constant darkness. The shape of these curves suggests first, a primary reaction to some force or stimulus followed by a failure to maintain this response, and second, the occurrence of a reaction in an opposite direction which, it may be conjectured, was due either to a simple readjustment or to the operation of a dissimilar stimulus. A second type of curve is characterized by a progressive downward trend with little or no tendency toward a final reversal of this movement, as is shown by the results obtained with such organs as the liver, the kidneys, and the hypophysis of the

light groups and the lymph nodes of the two experimental and the inside control series. It would appear that the causative factor or factors responsible for this type of variation in organ weight were operative throughout the period of observation, that these particular organs were capable of a continued response in a like manner during this time, and that, in addition, no force capable of eliciting a response in a different direction was effective in changing the organ weight reaction. The third type of curve resembles the first except that there is a tendency for it to begin on an approximately horizontal level, as illustrated by the curves for the thyroid of the light and dark series and for the parathyroids and suprarenals of the inside controls and the dark groups. The shape of these curves suggests an initial response to some cause which either ceased to be effective in this respect or was no longer operative, or else that another causative factor acting in the opposite direction superseded or effectively opposed the first. These examples indicate the complex nature of the relationship between physical environment on the one hand and the animal organism on the other. But, with due regard for other factors which may enter into this relationship, the general types of the curves obtained in this experiment are corroborative evidence of a weight reaction on the part of the organs of the body in response to the influence of external forces as represented by physical environment.

The observations recorded in this study bring out the significant fact that the variations in organ weight associated with the artificial environments employed all fell within the so called normal limits as determined by a study of 645 stock rabbits (4). From the point of view of organic reaction, therefore, our experimental conditions must be considered as being within the scope of action of the physical factors which ordinarily contribute to the occurrence of spontaneous variations of organ weight. It is noteworthy, however, that the variations which did occur tended to approach the extreme limits of spontaneous variation and to persist there.

An interesting feature of this experiment was the character of organ weights of the groups of rabbits kept in an unaltered room as contrasted with those recently brought to the laboratory. In general, the weights of organs per kilo of body weight were somewhat smaller and more uniform in the indoor controls, and with certain organs,

the thymus and the lymph nodes in particular, this tendency appeared to be more marked with the longer periods of the experiment. A sedentary cage life with a regular and abundant food supply might be expected to affect the weights of certain organs and perhaps particularly those more immediately concerned with metabolic activities. The most pronounced weight diminution occurred in the main mass of mesenteric lymph nodes, but the superficial lymph nodes also showed almost as striking an effect. However, there were definite fluctuations of mean weight of other organs in the inside control series, and in the case of the thyroid, the parathyroids, and the liver, for example, pronounced variations were observed. While there is no doubt that conditions of indoor life may affect organ weight of rabbits, other influences such as age and breed must be considered before the relative importance of this factor can be estimated.[1]

Little is known either of the weight relationships of one organ to another in normal or diseased states or of the factors that may affect these relationships. It seems unwise, therefore, to carry the analysis of results further at the present time.

Finally, it should be pointed out that while these experimental results indicate that an environment of constant light having none of the shorter ultra-violet rays and one of practically constant exclusion of light were associated with and presumably were the cause of definite variations in the physical state of certain organs of normal rabbits, it cannot be assumed that similar effects, either of degree or even of kind, would necessarily be obtained even with the use of the same environments. A different state or condition of organs at the initiation of experimental procedures might operate toward the production of different results, while the effect of various forces during the experimental period cannot be predicted. It is not

[1] In connection with the effect of indoor cage life upon organ weight, it is of interest to note that the reaction to such an experimental disease as a transplantable malignant neoplasm is not necessarily the same in rabbits caged indoors for comparatively long periods and in rabbits which have been in the laboratory for only a few days prior to inoculation. In an experiment carried out in February and March, 1926, the malignancy of the tumor in recently acquired rabbits was considerably greater than in those animals which had been in the laboratory for several weeks.

unlikely that as our knowledge of the diverse factors which affect the mass and mass relationships of organs increases, some will be found manifesting effects comparable with those observed under the conditions of this experiment, while others will be associated with dissimilar effects. The results of any experiment of this type, therefore, will depend upon all the factors involved and the extent or degree to which their influence upon organ weight is manifested.

SUMMARY AND CONCLUSIONS.

An analysis has been made of the organ weights of normal rabbits exposed to a constant illumination having none of the shorter ultraviolet rays and of other rabbits kept in darkness for periods of 2 to 12 weeks.

The environment of constant illumination was associated with a well marked decrease in the relative weights of most organs, and in certain instances this occurred when the organ weights of the controls were becoming increasingly large. There was also an associated effect of stabilization of organ weight.

The majority of the organs of rabbits caged in constant darkness also showed a tendency toward decreased and stabilized weights, but these effects were less pronounced than in the rabbits caged under conditions of constant illumination. A notable exception to this general result was the weight of the liver which was markedly increased.

The results of this experiment support the conception that there is a relationship between light and the physical state of the animal organism which may be expressed in the concrete form implied by the trend or direction of organ weight.

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THE CHEMICAL NATURE OF SUBSTANCES REQUIRED FOR CELL MULTIPLICATION.

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It is well known that tissue cells live indefinitely when cultivated in plasma and embryonic juice.[1] In such a medium, strains of fibroblasts and epithelium have proliferated without interruption for several years. It appeared that the chemical substances from which the cells synthetize new protoplasm are constituents of embryo juice and not of blood serum.[2] However, it has not been ascertained whether the building up of new cells merely requires nutrient materials present in the embryo juice or whether the juice also contains some specific hormone essential to cell division. Many attempts have been made to determine the nature of the substances used by the tissues, and to isolate the principle responsible for growth. Embryonic tissue extracts were fractionated and the activity of each fraction was tested on fibroblasts. These experiments pointed out that the growth-promoting substance consisted of a protein or was closely associated with a protein.[3] The suggestion was made that the nitrogenous material required by the tissues might be a product obtained from the protein by the cells through some ferment action.[3] It was also found that amino acids and other dialyzable nitrogenous compounds of the embryonic juice caused no increase in the mass of the tissues, although they had the property of stimulating cell migra-

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755; *Compt. rend. Soc. biol.*, 1924, xc, 562; *J. Exp. Med.*, 1925, xli, 337.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387.

tion and multiplication.[4,5] The products of tryptic digestion and complete peptic digestion of embryo juice protein were also unable to support cell proliferation, under the conditions of our experiments.

Further investigation of the processes of cell nutrition led to unexpected results: tissue cells were found to utilize the higher cleavage products of the protein molecule for multiplication.[6,7] The purpose of the present paper is to report the experiments in the course of which this important fact was discovered.

Technique for Preparing the Protein Split Products.

The protein split products were obtained by digesting embryonic pulp, egg white, rabbit brain, and commercial fibrin presumably from ox blood, at 40°C. with Fairchild's pepsin in N/20 HCl. The process was carried out for different lengths of time, and the ratios of pepsin to protein and of protein and pepsin to acid were varied in such a manner as to obtain products representing different states of digestion. The degree of digestion was followed by amino nitrogen determinations. Afterwards, the different solutions were brought to a pH of 7.0 by the addition of normal NaOH, and boiled down to somewhat less than half their original volume, in order to destroy the pepsin and remove the toluene which was added in some instances as an antiseptic. The boiling was also designed to remove any unchanged protein or heat-precipitable products and to concentrate the salts. After determination of the cryoscopic point, the solutions were rendered isotonic by the addition of water or Ringer solution of five times its usual concentration. The pH was adjusted at 7.2 to 7.4, and the ratio of total nitrogen to amino nitrogen determined.

Split products were also obtained by autoclaving the protein in water, in N/10 HCl or NaOH in a steam sterilizer at various pressures for different lengths of time. The pH was then adjusted and the solution prepared as described above.

Technique for Testing the Growth-Promoting Action of the Protein Cleavage Products.

The growth-promoting action of the above substances at several concentrations was tested on fibroblasts, according to two techniques which have already been described. In the first procedure,[8] fragments of heart were cultivated on

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[4] Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

[5] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 397.

[6] Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060.

[7] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627.

[8] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

hollow slides in a control medium composed of equal parts of plasma and Ringer solution, and in an experimental medium composed of equal parts of plasma and the protein cleavage products. Two or three cultures were made in each of several different concentrations of the experimental material, and allowed to grow for 48 or 72 hours. The growth was measured by the width of the ring of new tissue, or by the increase in area of the colonies. In the second procedure,[9] heart tissue or fibroblasts were cultivated in flasks containing 0.5 cc. serum, 1 cc. Tyrode

TABLE I.

Rates of Growth of Fibroblasts in the Peptic Digestion Products of Embryo Tissue.

Culture group No.*	Chemical preparation No.	Digestion	Total nitrogen	Amino nitrogen	Total N / Amino N	Width† of new tissue					Time of growth	Ratio: Peptic digest / Ringer solution			
						Ringer solution	Peptic digest								
							Pure	Diluted				Pure	Diluted		
								1:2	1:4	1:8			1:2	1:4	1:8
		hrs.	*per cent*	*per cent*							*hrs.*				
3171 A	X–274	32	.378	.168	2.25	1.5	2.0	1.5	2.0		48	1.3	1.0	1.3	
8649 D‡	X–271	16	.298	.135	2.21	5.0				4.0	48				.8
8643 D	X–271	16	.298	.135	2.21	2.0	2.0	2.0	2.0		48	1.0	1.0	1.0	
3178 A§	X–276	3.5	.352	.0525	6.7	1.5	5.0–6.0		4.0	5.0	48	3.6		2.6	3.3
3185 A§	X–276	3.5	.352	.0525	6.7	3.0	7.0		9.0	6.0	72	2.3		3.0	2.0
3192 A	X–276	3.5	.352	.0525	6.7	2.0	2.0		4.0	3.0	48	1.0		2.0	1.5
3201 A‖	X–287	3.5	.358	.0825	4.34	1.5	6.0		2.5	2.0	48	4.0		1.7	1.3
3219 A‖	X–287	3.5	.358	.0825	4.34	3.0	7.0		3.0	2.5	48	2.3		1.0	.83

*Each culture group number represents 6 to 10 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Experiments were also made at dilutions of 1:12 and 1:16. These showed no increase over the control.

§ Tyrode solution was used as control in place of Ringer solution.

‖ A second control solution, containing pepsin treated in the same way as the experiment, was used. The cultures in the control pepsin solution showed less growth than those in the control Ringer solution.

solution, and 0.5 cc. embryonic tissue extract. After coagulation, 1 cc. of Tyrode solution was added to the controls and 1 cc. of the preparation to the experiments. Every 2 days, the fluid medium was removed, the coagulum washed with 2 cc. Tyrode solution, and fresh fluid added. When digestion of the fibrin occurred, the coagulum of both control and experiment was patched with 0.25 cc. plasma and 0.25 cc. embryonic extract. The tissues were allowed to grow for periods

[9] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

varying from 1 to 2 weeks. The cultures were placed under the projectoscope daily, and the area of the colonies was traced and measured with the planimeter. The action of the experimental substances was expressed by a graph on which the area of the colonies was plotted as a function of the time.

Action on Fibroblasts of Embryo Tissue Hydrolyzed by Pepsin.

Embryonic tissues reduced to a fine pulp in a Latapie apparatus were digested with pepsin for 32, 16, and 3.5 hours respectively, and a comparison was made of the effects of these products and of Ringer solution on fibroblasts migrating from heart tissue. The products of 32 and 16 hours digestion showed no growth-stimulating action (Table I), and appeared to be somewhat toxic, the cells generally becoming loaded with fat granules and losing their activity. On the contrary, those obtained by 3.5 hours digestion showed a decided stimulating action, the width of the new growth being two and occasionally three times as great as that of the control in Ringer solution (Table I). The ratio of total to amino nitrogen was determined in each of these digests. The 32 and 16 hour digests had a ratio of 2.25 and 2.21 respectively, while the ratios in two different 3.5 hour digests were 4.34 and 6.7. The first two were slightly toxic, while the last ones promoted tissue growth, the greater growth being observed in the last digest. This indicated that the tissues utilized certain intermediate cleavage products for growth, probably some of the larger fragments of the protein molecule.

Action on Fibroblasts of Egg White Hydrolyzed by Pepsin.

The products of partial peptic hydrolysis of egg white were also tested on fibroblasts. The length of the time of digestion varied, but the protein was never completely hydrolyzed and some of the larger cleavage products remained in solution. These substances always determined a large growth of fibroblasts (Table II). It was thus obvious that nutrient substances could be obtained from egg white by pepsin hydrolysis.

Action on Fibroblasts of the Cleavage Products of Commercial Fibrin.

A similar effect on fibroblasts was exhibited by the cleavage products of a protein unrelated to that of chicken embryo or egg white, namely,

commercial fibrin (Table III). The growth-promoting action of some of the hydrolyzed products of fibrin was even greater than that of the embryonic tissue and egg white digests. This phenomenon may be due merely to the state of digestion reached by the protein, rather than to its nature. The split products of fibrin not only brought about

TABLE II.

Rate of Growth of Fibroblasts in the Peptic Digestion Products of Egg White.

Culture group No.*	Chemical preparation No.	Width† of new tissue					Time of growth	Ratio: Peptic digest / Ringer solution			
		Ringer solution	Peptic digest					Pure	Diluted		
			Pure	Diluted							
				1:2	1:4	1:8			1:2	1:4	1:8
							hrs.				
8667 D	X-288	2.3	2.5–4.0	4.0	2.5		48	1.1–1.7	1.7	1.1	
8673 D	X-282	3.0	5.0	4.0	3.0		48	1.7	1.3	1.0	
8680 D	X-288	1.5	2.0				48	1.3			
	X-288 a	1.5	3.0				48	2.0			
	X-291	1.5	3.5				48	2.3			
	X-291 a	1.5	2.5				48	1.7			
8702 D	X-305	3.0	6.0	4.0	3.0		48	2.0	1.3	1.0	
3177 A‡	X-275	1.5	4.0		4.0	3.0	48	2.6		2.6	2.0
3184 A‡	X-275	3.0	11.0		10.0	9.0	72	3.6		3.3	3.0
3191 A‡	X-275	2.0	5.0		3.0	3.0	48	2.5		1.5	1.5
3196 A	X-282	1.5	2.5	2.5	2.0		48	1.6	1.6	1.3	
8686 D	X-288	2.5	2.5				48	1.0			
	X-291	2.5	5.0				48	2.0			
	X-292	2.5	6.0				48	2.4			
	X-282	2.5	6.0				48	2.4			
3209 A	X-291	4.0	8.0		9.0	5.0	72	2.0		2.2	1.2
3212 A	X-292	2.0	4.0	6.0			48	2.0	3.0		

* Each culture group number represents 8 to 10 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Tyrode solution was used as control in place of Ringer solution.

a great increase in the proliferation of fibroblasts, but they also appeared to supply the tissues with the necessary nutrient material for prolonged growth (Fig. 1). When the digestion of fibrin was carried to the optimum degree, the growth of the tissues exceeded even that produced by embryonic juice. The fibroblasts that grew in the fibrin digestion products outlived those cultivated in embryo juice and, in

some cases, the volume of the tissue became four times as large as that of the controls (Fig. 3). Colonies of fibroblasts 2 cm. in diameter were obtained.

TABLE III.

Rate of Growth of Fibroblasts in the Peptic Digestion Products of Commercial Fibrin.

Culture group No.*	Chemical preparation No.	Width† of new tissue				Time of growth	Ratio: Peptic digest / Ringer solution		
		Ringer solution	Peptic digest				Peptic digest		
			Pure	Diluted			Pure	Diluted	
				1:2	1:4			1:2	1:4
						hrs.			
8682 D	X-297	3.0	6.0	10.0	12.0	72	2.0	3.3	4.0
8693 D	X-297	2.5	6.0	5.0	6.0	48	2.4	2.0	2.4
3232 A	X-297	3.0	5.0	5.0		48	1.7	1.7	
3225 A	X-297	2.0	5.0	3.0	3.0	48	2.5	1.5	1.5
8683 D	X-298	2.0	3.0	3.0	3.0	48	1.5	1.5	1.5
8692 D	X-298	2.0	4.0	6.0	6.0	48	2.0	3.0	3.0
8703 D	X-298	2.5		5.0		48		2.0	
	X-299	2.5		6.0		48		2.4	
3233 A	X-298	2.0	4.5	3.5		48	2.25	1.75	
8703 D	X-299	4.0		6.0		48		1.5	
3234 A	X-299	4.0	5.0	5.0	6.0	48	1.25	1.25	1.5
8696 D	X-303	4.0	7.0	8.0	5.0	48	1.75	2.0	1.25
3288 A	X-329	2.0	6.0			48	3.0		
8710 D	X-310	2.0	3.0	3.5	3.0	48	1.5	1.75	1.5
3265 A	X-318	1.5	4.0			48	2.6		
	X-302		4.0			48	2.6		
	X-304		4.0			48	2.6		
3277 A‡	X-324	2.5	4.5			48	1.8		
	X-325		6.0			48	2.4		
	X-326		6.0			48	2.4		
8732 D‡	X-324	1.0	1.5			48	1.5		
	X-325		4.5			48	4.5		
	X-326		6.0			48	6.0		

* Each culture group number represents 8 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Preparations X-324, X-325, and X-326 varied considerably in the degree of hydrolysis. The ratio of total to amino nitrogen was 5.2, 6.3, and 9.0 respectively.

In order to secure some information concerning the nature of protein cleavage products utilized by the tissues, three different digests were prepared by varying the ratio of the weight of fibrin to the weight

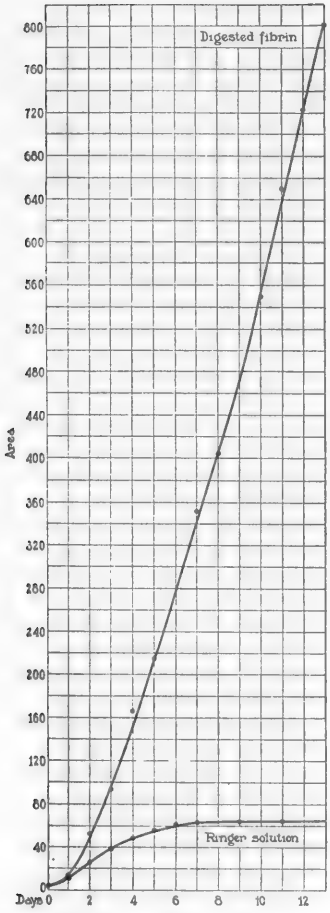

FIG. 1.

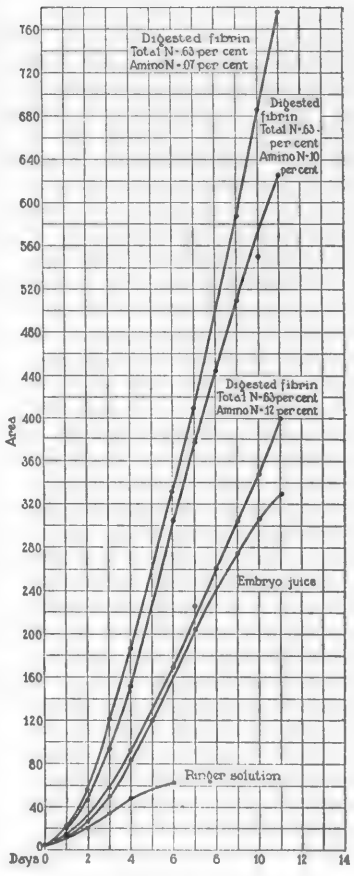

FIG. 2.

FIG. 1. Experiment 3284 A. Growth of fibroblasts in the products of partial peptic digestion of commercial fibrin. The control in Ringer solution died in 7 days. At the end of 2 weeks, the cells cultivated in the digested fibrin were still proliferating rapidly.

FIG. 2. Experiment 3287 A. Comparison of the rate of growth of fibroblasts in three fibrin digests of different degrees of hydrolysis, and in embryo juice and Ringer solution. The three fibrin digests contained the same concentration of total nitrogen, but the ratios of total to amino nitrogen were 9.0, 6.3, and 5.2 respectively. The growth of the cultures in the fibrin digests continued far beyond the points shown in this chart.

of pepsin. 5, 10, and 15 gm. of fibrin were digested in 200 cc. of N/20 HCl with 1 gm. each of Fairchild's pepsin. In the first case, all the fibrin was transformed into soluble products which remained in solution on neutralizing and boiling. In the other two cases, some fibrin was left and much protein and meta-protein were precipitated

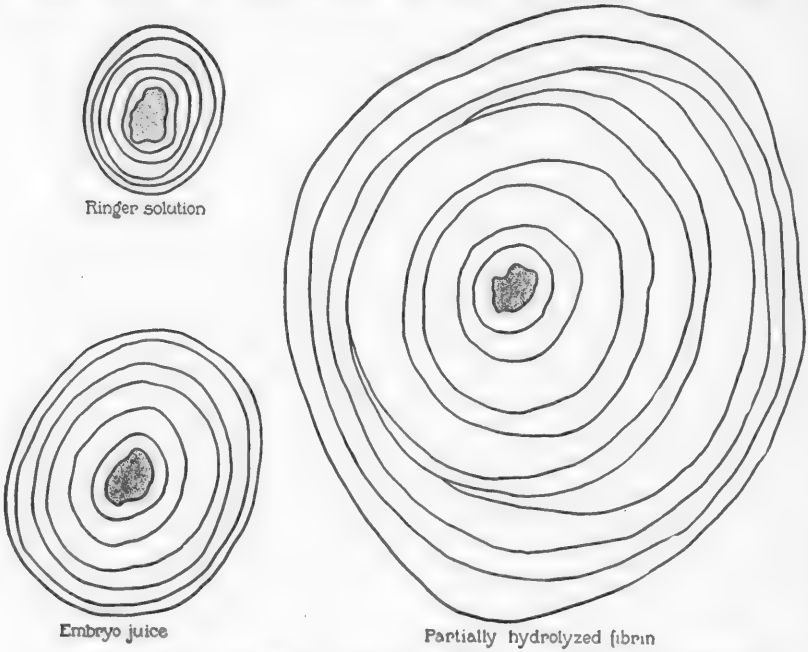

FIG. 3. Experiment 3287 A. Daily tracings under the projectoscope of areas of colonies of fibroblasts cultivated in Ringer solution, embryo juice, and partially digested fibrin. Some contraction of the clot took place on one side of the colony, resulting in a deformation. The growth in partially digested fibrin yielded the largest single colony of fibroblasts ever obtained *in vitro* in this laboratory.

when the solutions were neutralized and boiled. As a basis of comparison, the solutions were diluted to the same concentration of total nitrogen, 0.63 per cent, before being tested for growth-stimulating action on fibroblasts. The amino nitrogen concentration in these solutions was 0.12, 0.10, and 0.07 gm. per 100 cc., giving a ratio of

total to amino nitrogen of 5.2, 6.3, and 9.0, respectively. These solutions were compared with each other and with Ringer solution and embryonic tissue extract, with respect to their growth-promoting power on fibroblasts (Fig. 2). The results clearly indicate that the higher cleavage products are the ones utilized by the tissues. All of these digests produced more rapid and prolonged growth than the embryo juice did. Another pair of digests was compared in which the ratio of total to amino nitrogen was 7.7 and 8.6. In this case, a similar result was obtained. A series having the ratios 8.4, 8.6, and 10.3 showed very little difference, the rate of growth being exceptionally large in all three.

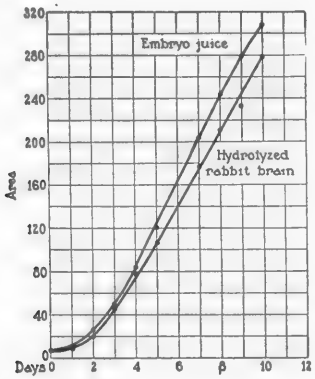

FIG. 4. Experiment 3304 A. Growth of fibroblasts in rabbit brain partially hydrolyzed by pepsin. The growth is comparable to that in embryo juice.

Action on Fibroblasts of Other Proteolytic Products.

Various proteins when partially hydrolyzed appeared to be nutrient for fibroblasts. Two pepsin digests of rabbit brain showed a nutrient action almost equal to that of embryo juice (Fig. 4). These digests gave a strong biuret reaction and contained protein split products having a ratio of total to amino nitrogen of 5.3. The rate of growth produced was comparable to, though less than, that of the fibrin digest having a ratio of 5.2. Hydrolytic products obtained by autoclaving egg white and fibrin in an acid medium at 15 pounds pressure for 1.5 hours proved stimulating to growth (Table IV). More

complete hydrolysis of these substances produced by autoclaving at 25 pounds pressure for 4 hours yielded toxic or inactive products, thus affording a further indication that the higher cleavage products of protein determine growth.

Action on Fibroblasts of Commercial Peptones.

Commercial "peptones" in solutions from 20 to 1 per cent, sterilized by boiling, were used in the culture medium of fibroblasts.

TABLE IV.

*Rate of Growth of Fibroblasts in Protein Cleavage Products Produced by Autoclaving in Acid.**

Culture group No.†	Chemical preparation No.	Substance autoclaved	Width‡ of new tissue				Time of growth	Ratio: Experiment / Control		
			Ringer solution	Experiment						
				Pure	Diluted			Pure	Diluted	
					1:2	1:4			1:2	1:4
							hrs.			
3218 A	X-295	Fibrin	1.5	3.0			48	2.0		
3227 A	X-296	"	3.5	5.0			48	1.4		
3235 A	X-296	"	3.0	6.0			48	2.0		
3237 A	X-296	"	1.0	4.0			48	4.0		
3245 A	X-296	"	1.0	4.0			48	4.0		
8689 D	X-296	"	2.0	4.0			48	2.0		
3205 A	X-290	Egg white	2.0	4.0	3.0	3.0	48	2.0	1.5	1.5
3205 A	X-290	" "	2.0	8.0	8.0	9.0	72	4.0	4.0	4.5

* 15 pounds pressure for 1.5 hours.

† Each culture group number represents 4 to 8 experiments.

‡ Measured under the microscope in 0.1 mm. units.

Armour's peptone was almost inactive. Parke Davis' and Fairchild's peptones exhibited a small growth-promoting action, while Witte's peptone determined a large growth of fibroblasts (Fig. 5). At a concentration of 5 to 10 per cent, it was toxic. Even in the cultures containing a low concentration of the peptone, the cells showed many fat globules. In spite of this, the rate of growth of the tissues treated with Witte's peptone was as great as that cultivated in embryo juice (Fig. 6). This difference in activity of the commercial peptones is undoubtedly due to the very large concentration of proteoses and the

smaller amount of the lower degradation products present in Witte's peptone in comparison with the others.

Action on Fibroblasts of the Proteose Fractions of Witte's Peptone.

The foregoing experiments indicated that proteoses are probably responsible for the effect of Witte's peptone and other protein digests on the proliferation of fibroblasts. A few preliminary experiments on the fractionation of Witte's peptone confirmed this idea. The meta-

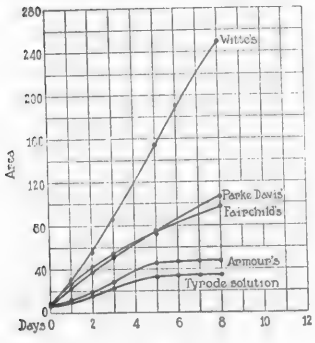

FIG. 5.

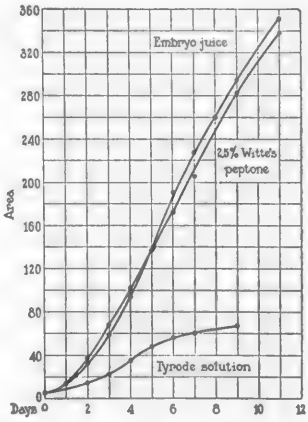

FIG. 6.

FIG. 5. Experiment 3349 A. Action of 2 per cent solutions of commercial "peptones" on the growth of fibroblasts *in vitro*.

FIG. 6. Experiment 3133 A. Growth of fibroblasts in 2.5 per cent Witte's peptone, the control being cultivated in embryo juice.

protein precipitated at pH 6.0 and the substances precipitated by 2.5 per cent trichloracetic acid had some growth-stimulating action on fibroblasts, but not as much as the remaining fractions. A fraction containing only proteoses was, therefore, prepared by precipitating the higher products, including a part of the proteoses, in 2.5 per cent trichloracetic acid. The remaining proteoses were separated from the peptones and lower degradation products by saturating at 33°C. with sodium sulfate, after removal of the trichloracetic acid. This process was repeated four times to insure removal of the other frac-

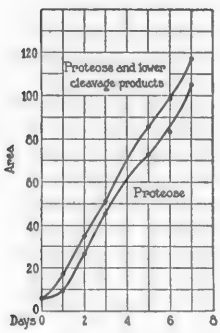

FIG. 7.

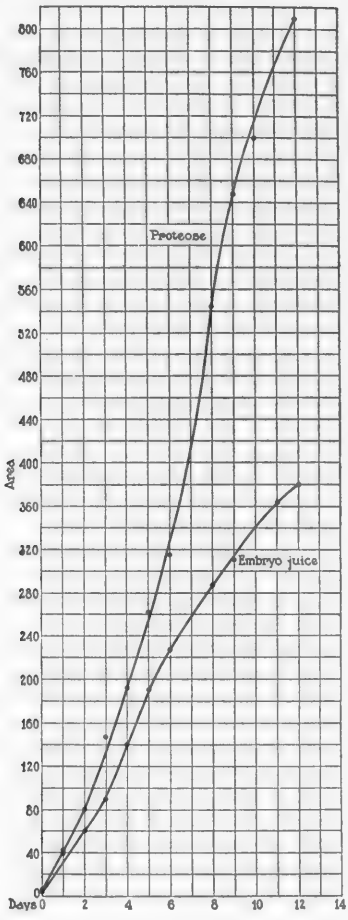

FIG. 8.

FIG. 7. Experiment 3292 A. Comparison of the rate of growth of fibroblasts cultivated in the proteose fraction of Witte's peptone and in the fraction containing proteoses, peptones, and lower degradation products. The cells in the proteose were in better condition than those in the fraction containing the lower split products, and they multiplied at about the same rate. Concentration of nitrogen equaled 0.398 per cent in both media.

FIG. 8. Experiment 8758 D. Growth of fibroblasts in the pure proteose fraction of Witte's peptone (0.2 per cent N). The control culture in embryo juice died on the 13th day. The colonies in the proteose were still growing after 30 days cultivation.

tions. The sulfate was precipitated by barium chloride and the solution was finally dialyzed. The effects on fibroblasts of this preparation at 0.398 per cent nitrogen, and of the trichloracetic acid filtrate containing the proteoses, peptones, and smaller split products, at the same nitrogen concentration, were compared. The rates of growth in the two media did not differ appreciably (Fig. 7), but the cells cultivated in the purified proteose solution showed fewer fat globules in their cytoplasm than those kept in the solution where peptones were still present. In another experiment, a comparison was made of the tissues growing in the purified proteose solution at 0.2 per cent N and in embryonic juice (Fig. 8). The colonies kept in the purified

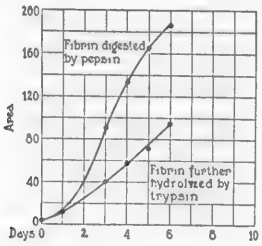

FIG. 9. Experiment 3326 A. Growth of fibroblasts in fibrin partially digested by pepsin and in the same substance further digested by trypsin at equal nitrogen concentration.

proteose grew twice as rapidly as those cultivated in embryonic tissue extract, and the cells contained a smaller number of fat globules.

Evidently, the tissues utilize proteose for building new protoplasm. The concentration of nitrogen in the proteose solutions and in the various protein digests is larger than that of embryonic tissue extract. This undoubtedly means that a part of these nitrogenous substances is not utilized by the tissues. Further fractionation of the proteose is being carried out in order to ascertain whether a more active moiety can be obtained.

Action on Fibroblasts of the Tryptic Digest of the Protein Cleavage Products.

A comparison was made of the rates of growth of fibroblasts in the most active peptic digest of fibrin and in the same solution further

hydrolyzed by trypsin. The results (Fig. 9) demonstrated that hydrolysis by trypsin greatly reduced the growth-promoting power of the solution, although it did not destroy it completely. The tryptic hydrolysis was not complete, however. It reduced the ratio of total to amino nitrogen from 9.0 to 2.5. Analysis of this tryptic digest showed the absence of proteose except in the smallest traces, and the presence of a considerable amount of peptones and peptides. Therefore, it would seem that the peptones and possibly some of the lower degradation products are nutrient to a certain extent, although they

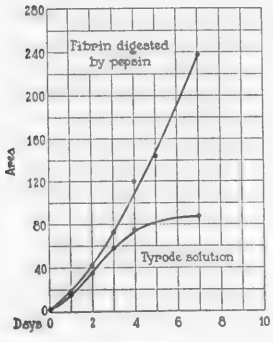

FIG. 10.

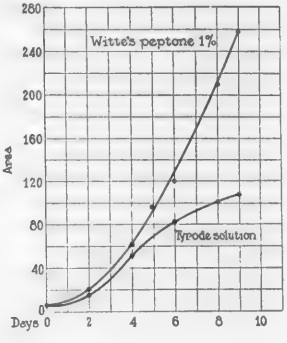

FIG. 11.

FIG. 10. Experiment 3283 A. Growth of a 14 year old strain of fibroblasts in the products of partial peptic digestion of fibrin. Control tissue in Tyrode solution.

FIG. 11. Experiment 8722 D. Growth of a 14 year old strain of fibroblasts in 1 per cent Witte's peptone. Control tissue in Tyrode solution.

do not promote the rapid growth which is produced by the proteoses. All of the amino acids were not present in this tryptic digest in the same concentration as in the peptic digest containing proteoses, for some insoluble products were formed as a result of the hydrolysis. This suggests the hypothesis that the function of the proteose is to furnish a higher concentration of certain amino acids than could be obtained even from their saturated solutions, and to supply them to the tissue cells in a soluble and diffusible form.

Action of the Protein Split Products on Pure Strains of Fibroblasts, Leucocytes, Epithelium, and Thyroid Cells.

The foregoing experiments were generally made on fibroblasts that had migrated from fragments of embryonic heart. Similar experiments were repeated on a 14 year old strain of fibroblasts and on pure cultures of pavement epithelium and thyroid cells. Fibroblasts obtained from the old strain were cultivated in various digestion products of fibrin, in Witte's peptone, and in fractions of Witte's peptone (Figs. 10 and 11). The results were similar to those reported above.

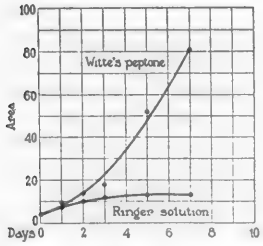

FIG. 12.

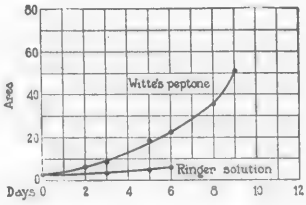

FIG. 13.

FIG. 12. Experiment 3337 C. Growth of iris epithelium in Witte's peptone. Control tissue in Ringer solution.

FIG. 13. Experiment 3216 A. Growth of guinea pig skin in Witte's peptone. Control tissue in Ringer solution.

Epithelial and thyroid cells as well as fibroblasts were found to multiply with great speed in the presence of the protein split products (Figs. 12 to 14). Leucocytes died when kept in Witte's peptone at a concentration of 2.5 per cent. They utilized it, however, for their nutrition when its concentration in the medium was much lower.

DISCUSSION AND CONCLUSIONS.

These results indicate the nature of the nitrogenous substances required by fibroblasts and epithelial cells for the building up of protoplasm. Tissue cells obtain their nitrogen from proteoses and possibly from some of the other split products of the proteins. The smaller split products do not induce the rapid proliferation of the cells that the

proteoses do, although they appear to be utilized to a slight extent as nutrient materials. The rôle of amino acids, peptones, and proteoses in cell nutrition has not been studied extensively. Burrows[10] found that certain amino acids, even at a low dilution, were toxic for tissues growing *in vitro*. Philippson and Mendeleef[11] hydrolyzed serum by sulfuric acid and observed that the tissues displayed more activity in hydrolyzed than in normal serum. Unfortunately, the technique of those experimenters was imperfect, and their results did not indicate whether the medium was nutrient or merely stimulating, and whether the effect could be attributed to a loss of the natural inhibiting power of the serum or to the production in this serum of nutrient or stimulat-

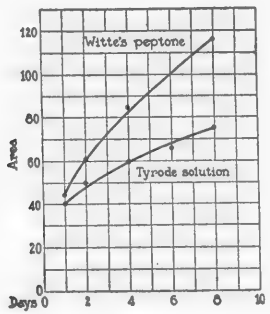

FIG. 14. Experiment 3526 C. Growth of thyroid cells in Witte's peptone. Control tissue in Tyrode solution.

ing substances. Carrel and Ebeling[4] studied in a quantitative manner the action of artificial mixtures of amino acids on fibroblasts in pure culture, and found that these acids were not toxic and brought about some acceleration of the cell migration without increasing, however, the mass of the tissues. The ultrafiltrate of embryonic juice yielded a similar result, when added to the medium of fibroblasts. These facts have been confirmed lately by Wright,[12] who observed the presence in

[10] Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

[11] Philippson, M., and Mendeleef, P., *Soc. roy. Sc. méd. et nat. Bruxelles, Vol. Jubilaire*, 1822–1922, 713.

[12] Wright, G. P., *J. Exp. Med.*, 1926, xliii, 591.

embryonic juice of substances which pass through a collodion membrane and stimulate cell division. So far, no increase in the mass of a tissue in pure culture has been determined by a mixture of amino acids, or by the ultrafiltrate of embryonic juice. On the contrary, the larger fragments of the protein molecules can be used as food by tissue cells.

These facts agree with the results previously obtained by the fractionation of embryo juice.[3] In those experiments, it was found that fibroblasts do not feed upon the amino acids and other ultrafilterable constituents of the embryo juice, but on the protein fraction. It might be supposed that the protein was utilized as such by the cells, or was

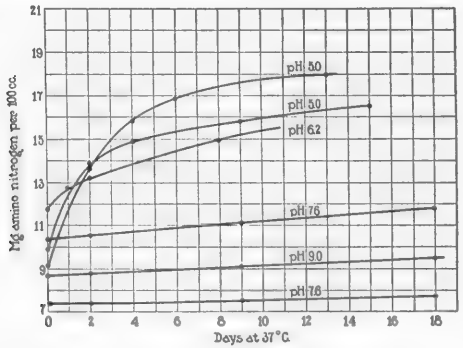

FIG. 15. Increase of amino nitrogen at pH 5.0 and 6.2 in embryo juice incubated at 37°C., showing the presence of a proteolytic enzyme.

hydrolyzed before being absorbed. Many attempts were made to obtain a fraction more active than the original embryo juice. The results of these experiments were negative, probably because the growth-activating substances are not preformed in the embryo juice but are continuously made from its proteins by the cells themselves or by an enzyme contained in the medium. It is a plausible hypothesis that an enzyme contained in the tissue juice becomes active in the presence of living cells. When embryonic tissue juice is incubated at 37°C., at a pH varying from 7.6 to 9.0, no increase in amino nitrogen is observed. But the quantity of amino nitrogen increases rapidly as the medium is made more acid (Fig. 15). At pH 6.2, some hydrolysis

takes place and an increasing amount at pH 5.0. It must be remembered that the fluid contained in the flasks is at a pH of 7.6 and so buffered that it does not change to any great extent during the period of incubation. Under these conditions, the embryo juice enzymes do not manifest their activity, and no proteoses are produced. The pH of the coagulum immediately adjacent to the tissue, however, is modified by the mere presence of living cells. Long ago, Rous observed that tissues growing in a medium containing litmus became surrounded by a pink area.[13] When phenol red was added to the coagulum in the flasks, the medium as a whole remained alkaline, but a considerable acid production took place around the colonies as shown by the brilliant yellow crown surrounding them. A change to a pH of at least 6.0 occurred at the surface of the cells, which is probably sufficient to enable the tissue juice enzymes to hydrolyze proteins into the proteoses which are absorbed and utilized by the tissues. The objection may be made to this hypothesis that serum is not nutrient for fibroblasts, although it contains enzymes which hydrolyze the proteins in an acid medium. However, it is possible that the removal of the antiproteolytic and growth-inhibiting lipoids would cause serum proteins to be used by the cells, provided a sufficient concentration of enzyme remained. Or it may be that the configuration of the molecules of the protein of embryonic juice is such that they are attacked by cell enzymes which have no effect on plasma proteins.

It has been considered as probable that embryonic juice contains, together with nutrient substances, a specific hormone essential to cell division. The fractionation of the juice did not give any evidence of the existence of such a hormone. Although amino acids or dialysate products had a stimulating effect on cell migration and multiplication,[4, 12] the substances found to support continuous growth consisted of proteins. The experiments described in the present article render it probable that for cell multiplication a specific hormone is not required and is not contained in embryonic juice, since growth-promoting substances can be obtained from proteins of many sources. While proteoses undoubtedly furnish the nitrogenous materials required for cell multiplication, the fact remains that these products

[13] Rous, P., *J. Exp. Med.*, 1913, xviii, 183.

have been obtained from impure proteins and, therefore, other substances may be present which have some action on tissue growth. The study now under way of the digests of pure proteins may show what other products, if any, beside the proteoses are essential to the proliferation of epithelial cells and fibroblasts.[14]

SUMMARY.

1. Fibroblasts and epithelial cells in pure culture obtain the nitrogen, which they build into protoplasm, from proteoses and possibly other primary derivatives of proteins. These proteoses have been prepared from embryo tissues, egg white, commercial fibrin, rabbit brain, Witte's peptone, etc.

2. The presence in embryo juice of a hormone that stimulates cell division is improbable.

3. Proteoses separated from peptic digests of fibrin by sodium sulfate determine a more abundant and prolonged multiplication of the fibroblasts than is produced by embryo juice. Peptones and the smaller split products appear to furnish some nutrient material, but do not cause the rapid proliferation characteristic of proteoses, and are sometimes toxic for tissue cells.

4. Possibly the effect of embryo juice on fibroblasts and epithelium is due to the splitting of the protein of the juice into proteoses by the cell enzymes, or by other enzymes activated by the presence of living cells.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

[14] Since this paper was written, a study has been begun of the products of the partial peptic digestion of crystalline egg albumin. The albumin was recrystallized three times to insure removal of all other substances. A peptic digest of this material, having a ratio of total to amino nitrogen of 4.7, was found to promote the growth of fibroblasts in the same manner as the proteolytic products of the impure proteins. This fact demonstrates that the action of the peptic digests reported above is due to the higher protein split products, and not to any accompanying impurity.

AUTOPLASTIC THYMUS TRANSPLANTS.

II. With Particular Reference to the Regeneration of the Reticulum Cells and the Formation of Hassall's Corpuscles.

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Plates 17 and 18.

(Received for publication, June 4, 1926.)

In spite of the great number of studies dealing with the thymus gland, no unanimity of opinion has been reached in regard to the formation and fate of Hassall's corpuscles. We are reporting the facts concerning the formation of these corpuscles as we have found them by studying the regeneration of autoplastic transplants in the guinea pig. This animal's thymus normally contains large numbers of Hassall's corpuscles and is therefore suitable material for such a study.

A review of the thymus transplant literature, for which the reader is referred to a previous paper (1), shows that this gland has been successfully autotransplanted subcutaneously, intramuscularly, subperitoneally, intraperitoneally, and into the spleen. Previous studies of thymus transplants have yielded little information concerning the thymic elements, among them Hassall's corpuscles, because the regeneration and growth of the transplants were not traced in detail.

The purely morphological literature dealing with Hassall's corpuscles is extensive and will not be summarized. Numerous theories concerning their origin have been developed since 1849 when Hassall first described them (2), but he did not discuss their mode of origin. In recent years two conceptions concerning the origin of these bodies have been recognized:

First. Schambacher advanced the theory that Hassall's corpuscles when fully developed represent the remains of the original endodermal thymic cords and tubules (ducts of Remak), now involuted and hyalinized (3).

Second. Hammar elaborated the theory that Hassall's corpuscles are developed

by proliferation of the thymus reticulum cells (4). These cells are endodermal in origin and are formed by a transformation of the epithelial cells of the original thymic ducts to a ramified form. Hassall's corpuscles first appear in the human thymus at about the 3rd month of embryonal life.

Methods.

Young guinea pigs from 30 to 45 days of age were operated upon under ether anesthesia, with strict asepsis. To remove the thymus, a midline incision was made extending from the angle of the lower jaw to the manubrium, the fascia was incised, and both lobes of the gland were removed and immediately placed in sterile physiological salt solution kept at about 38°C. The abdominal wall was then prepared for transplantation by making a midline longitudinal skin incision, reflecting the skin, and with a cataract knife making large pockets between the muscle and fascia. The surrounding fat and areolar tissue were dissected from the thymus and each lobe was introduced into a pocket, the mouth of which was then closed with a silk ligature.

The transplants when removed were immediately fixed in Zenker-formol or Bouin's solutions. The tissues were embedded in paraffin, cut serially, and stained with hematoxylin-eosin. Zenker-formol fixation was not as satisfactory as Bouin's for obtaining good pictures of reticulum cells and mitotic figures.

Histological Findings.

Transplants were studied at 24 hour intervals from the 1st to the 14th day, the subsequent stages being 16, 21, 30, 62, and 120 days. The histological appearance of the transplant for a given day will vary with many factors, such as the size of the lobe transplanted, its vascularization, the thickness of the transplant in the region through which the section was taken, and the age of the animal. Therefore the interpretation of the histological picture necessitates consideration of these factors, and a composite picture of the successive stages of degeneration, regeneration, and growth of the thymus can be drawn only after a study of many serially cut transplants. On the whole, the conditions are as follows:

1 Day.—The thymic lobules are large, and the transplant is degenerating. A peripheral zone of thymus cells 10 to 12 rows deep, still quite normal in histological appearance, remains. The reticulum cells in this zone are actively phagocytic, and are ingesting the neighboring small thymic cells. The most pronounced degenerative changes are seen in Hassall's corpuscles, which show diminished staining and disappearance of the nuclei, and fragmentation of the bodies. The round thymic and reticulum cells in the central part of the transplant show ir-

regular clumping with pycnosis and caryorrhexis of the nuclei. The vessels of the pocket show hypertrophy of the endothelial cells and are engorged; some veins contain an excess of leucocytes and mononuclear phagocytes; the lymph vessels although engorged contain few lymphocytes. Between the pocket and the transplant there is edema, fibroblastic proliferation, and fibrin with leucocytes and mononuclears embedded in its meshes. Numerous capillaries have developed about the transplant and are penetrating its peripheral zone and vascularizing it. New vessels are also extending in between the lobules of the thymus. No mitotic figures are seen.

2 Days.—Many more new blood vessels have formed about the transplant and the peripheral zone is well vascularized. Here hypertrophied reticulum cells are phagocytosing the small thymic cells, after which they join the large central mass of debris, which now shows greater clumping and agglutination than in the previous stage. All the Hassall's corpuscles in the transplanted tissue have undergone complete degeneration.

3 Days.—The histological picture is essentially like that of the 2nd day, but with greater hypertrophy and phagocytic activity of the peripheral reticulum cells and progressive vascularization of the transplant.

4 Days.—Active regeneration has begun in the peripheral zone which has become much widened through hyperplasia of the reticulum cells, many of which are dividing mitotically. This zone is in some places entirely devoid of thymic round cells, while in other places clumps of these cells with an occasional division figure are seen. Hassall's corpuscles now appear here as spherical bodies measuring up to 150 micra in diameter and extending through the tissue for several hundred micra. The core of such a body consists of a degenerated mass of pinkish staining protoplasmic and nuclear material, arranged somewhat concentrically in wavy strands. Many reticulum epithelial cells surround this core, and are sometimes massed acentrically because of rapid amitotic division at one point.

These Hassall bodies are formed when clumps of actively hyperplastic reticulum cells are spent and degenerate. Concomitantly the surrounding reticulum cells proliferate and clothe the degenerating cells which eventually become the core of the Hassall's body. If the proliferation is acentric, a giant cell-like structure may be formed about the core. These bodies are true Hassall's corpuscles but atypical in histological appearance because they are produced very rapidly.

5 Days.—The reticulum cells are enlarged, polyhedral, and vacuolated. Numerous newly formed atypical Hassall's corpuscles are being added to the central mass of debris. Through active division *in situ* the small round thymic cells are increasing in number. Infiltration of these cells from the outside may be definitely excluded.

6 Days.—The changes are like those described for the 5th day only that the reticulum cell overgrowth has progressed. Compound atypical Hassall's corpuscles now appear as a result of the development of multiple areas of degeneration in the hyperplastic reticulum; these structures slowly join the central necrotic

mass, which has been reduced through phagocytosis. Between the regenerating lobules fibroblasts have appeared.

7 Days.—The transplant appears more lymphoid because of active mitosis of the small thymic cells.

8 and 9 Days.—Small irregular regenerated lobules of thymus appear, in which the lymphoid cells are multiplying by mitotic division. The centers of the lobules are occupied by single or conglomerate Hassall's bodies. When the larger ones are traced serially they extend through the lobules for many hundred micra; their cores are degenerated reticulum epithelial cells. The interlobular spaces are organized by fatty connective tissue and numerous blood vessels have appeared.

10 Days.—In places the hypertrophy of the reticulum epithelial cells is so marked, that the picture resembles stratified skin epithelium; the atypical Hassall's corpuscles appear like the pearls of a malignant epithelioma.

11 Days.—The centers of the regenerating thymus lobules now consist of hypertrophic reticulum epithelial cells with numerous single and compound atypical Hassall's corpuscles, while the lymphoid appearing peripheries show numerous dividing small round cells.

12 to 14 Days.—The thymus lobules, although irregular and smaller than normal lobules, have taken on their final form, but central degenerated epithelial cores are still present.

16 Days.—The reticulum epithelial cells are diminished in number; the central degenerated masses are reduced in size and are passing from the lobules into the interlobular spaces. The first differentiation of the regenerated thymus into cortex and medulla appears.

21 Days.—Regeneration of the thymus is now complete. The lobules are lymphoid in appearance, with numerous dividing small thymic cells. True Hassall's corpuscles appear now, developed from the reticulum epithelial cells in the following way: A spent reticulum epithelial cell swells and the surrounding reticulum cells react by arranging themselves around it. These concentrically arranged reticulum cells hypertrophy in the effort to phagocytose the original injured cell. The process may continue until two or more layers of reticulum cells have been added. In this way the Hassall's corpuscle grows, and as it grows, the center degenerates. The greater the central degeneration, the more active is the response of the peripheral reticulum cells. However, in a 21 day transplant only small Hassall's corpuscles are seen, generally not more than 50 to 75 micra in diameter, and they consist as a rule of a central degenerated cell with a surrounding coat of one or two layers of proliferating epithelial cells.

30 Days.—The lobules are distinctly divided into cortical and medullary zones. The Hassall's corpuscles are larger, and some solid masses of reticulum epithelium are also seen.

62 Days.—The transplant is somewhat involuted and fat appears between the lobules. Hassall's bodies are either small, or large and multiple with central cores of debris in which polymorphonuclears and lymphocytes are embedded. These

leucocytes have been phagocytosed by the proliferating reticulum epithelial cells during the formation of the corpuscles and have not infiltrated these bodies subsequent to their formation.

120 Days.—The transplant is involuted and large areas of fat have appeared between the small lobules. The reticulum is prominent, and numerous Hassall's corpuscles appear—large atypical bodies similar to those present in a 4 day transplant, small well formed Hassall's bodies, and conglomerate Hassall's corpuscles with large central areas of debris.

Summary of the Findings.

When an entire thymic lobe is autoplastically transplanted into the abdominal wall of a young guinea pig, the immediate changes which take place are characterized by degenerative phenomena which begin within a few hours, and affect at this time all but a small peripheral zone of the transplant. The Hassall's corpuscles rapidly degenerate. Within 24 hours the reticulum cells in this peripheral zone hypertrophy, becoming phagocytic. Vascularization proceeds and many newly formed blood vessels surround and penetrate the transplant. By the 4th day the reticulum cells of the peripheral zone increase through hyperplasia, while the retained round cells in this zone are phagocytosed and greatly reduced in number. Small atypical Hassall's corpuscles now develop in the periphery when the rapidly increasing reticulum cells degenerate. The continued hyperplasia and hypertrophy of the reticulum cells render the transplant epithelioid in appearance and at this stage Hassall's bodies appear like the pearls of a malignant epithelioma. The central debris is removed by the phagocytic reticulum cells which are rapidly growing in from the periphery. The excess of reticulum tissue now undergoes degeneration forming conglomerate atypical Hassall's corpuscles. Simultaneously the remaining round cells in the periphery rapidly increase by mitosis. Regenerated thymic lobules are formed and their epithelioid centers show numerous large atypical Hassall's corpuscles which when traced serially extend through the lobules for many hundred micra. When the central debris is phagocytosed, the large epithelial Hassall's bodies pass from the lobules into the interlobular spaces. Finally, regeneration is complete; the lobules are differentiated into cortical and medullary zones, true Hassall's corpuscles appear as a result of the swelling of one or more reticulum cells with

hypertrophy of the surrounding reticulum cells. The study of an involuting transplant proves the common origin of the atypical and true Hassall's corpuscles, the former being produced when the degeneration of the reticulum epithelial cells is rapid, the latter when it is slow.

DISCUSSION.

Regeneration of the thymus has been studied by other methods.

Jonson investigated the effect of hunger and inanition on the thymus of young rabbits and found, as have others, that the gland rapidly involutes. During involution the gland appears epithelial, and all Hassall's corpuscles degenerate, disappearing in about 4 weeks. After 2 weeks of good nutrition Hassall's corpuscles reappear, but regeneration progresses slowly (5).

Rudberg studying the rabbit thymus following x-ray injury noted degeneration of the reticulum cells which frequently led to the formation of cyst-like structures (6). The mechanism of the formation of these cysts is probably analogous to that of the atypical conglomerate Hassall's corpuscles which appear in a regenerating transplant.

Regaud and Crémieu x-rayed the thymus of cats and found that during the regeneration Hassall's corpuscles reach their maximum development from 8 to 12 days after treatment when they are gigantic and occupy about one-half of the parenchyma (7). About the time the small thymic cells reappear, these gigantic Hassall's corpuscles rapidly diminish in size. From 25 to 30 days after x-ray treatment, regeneration of the thymus is complete when small typical Hassall's corpuscles appear in the medulla. That there should be such similarity between the changes which these authors observed in the rayed cats' thymus and those which we have observed in regenerating transplants is interesting.

Goldner showed that adrenalin, when injected in large doses over short periods of time or in small doses over long periods, caused involutionary changes in Hassall's corpuscles (8).

Fulci, studying the rabbit thymus following cauterization, described epithelial overgrowth during regeneration (9). He believed that both Hassall's corpuscles and the small thymic cells were derived from reticulum cells which he termed "*Mutterzellen*."

Concentric epithelial Hassall's bodies have been described in four recent reports of carcinoma of the thymus. Foot, who has traced the formation of these corpuscles, believes that they are degeneration products of the carcinomatous reticulum cells (10). The tumor which he described contained in the necrotic foci the outlines of innumerable degenerated Hassall's corpuscles, some of which occupied an entire low power field.

It may be gathered that large Hassall's bodies can be formed in the regenerating thymus after a variety of injuries. The common origin of the atypical and the true Hassall's bodies has been proved in this study and their common derivation from the reticulum epithelial cells has been demonstrated, the atypical corpuscle arising when the degeneration of these cells is very rapid.

For a proper understanding of the reticulum reaction of the thymus one must remember that the epithelial thymus anlage is not equivalent in all cases. In the majority of mammals the anlage is of purely endodermal origin, but in some species, as for example the mole and guinea pig, it is purely ectodermal, while in others, such as the pig, it is of mixed ecto-endodermal origin. These differences in the origin of the anlage are eventually reflected in the histological appearance of the formed gland, particularly of the reticulum, which in the guinea pig consists of large epithelial cells tending to form concentric bodies. Ducts are rarely seen. On the other hand, the reticulum cells of the rat thymus show little tendency to Hassall body formation, while ducts are frequently seen.

Cysts and ducts found in the fully developed thymus are either remains of the original primary tubules and cords or derivatives of the reticulum epithelial cells. The only relationship between these structures and Hassall's corpuscles is that the cells, from which both the ducts and Hassall's corpuscles develop, are derived from the secondary epithelial cords. The view that normal Hassall's corpuscles represent the atrophic and hyalinized remains of thymic epithelial tubules and cords is untenable since actual counts of Hassall's corpuscles show that there may be an absolute increase or decrease in their number under certain conditions; they may disappear completely from the thymus and then reappear (transplant); and calculations show that in involuted thymuses of old animals the number of Hassall's corpuscles may be as much as 7 times that observed at birth. The view that Hassall's corpuscles may be formed throughout life must be accepted.

The Function of the Reticulum Cells and of Hassall's Corpuscles.

The feeling has often been expressed that the thymus has no important secretion. After having approached the problem from various

angles, I find myself agreeing with this view. However, the thymus is physiologically active in the body, as evidenced by its regeneration following suprarenalectomy, delayed involution after gonadectomy, rapid involution during certain infectious diseases, and the presence of an enlarged gland in status thymicolymphaticus, Addison's, and Graves' diseases.

Concerning Hassall's bodies, I believe that the fully developed corpuscles have no function, being merely aggregates of spent reticulum cells with central cores resulting from degeneration of these cells. The proliferation of thymus reticulum cells, which results in the formation of these corpuscles, may be brought about when the phagocytic activity of the reticulum cells is stimulated by toxins, bacteria, or the presence of dead or injured cells. This fact is beautifully illustrated when the regeneration of a transplant of a whole lobe in the guinea pig is compared histologically with that of a small piece of thymus. The hyperplasia of the reticulum cells in the former is much more marked because of the greater necrosis which results in a more vigorous reticulum cell reaction, leading to the formation of more numerous and larger Hassall bodies.

A study of the progressive changes in the transplant suggests that the essential functions of the reticulum cells are proliferation and phagocytosis under the influence of noxious substances. When these reacting reticulum cells degenerate, the surrounding reticulum cells proliferate, and Hassall's corpuscles with degenerated cores are formed.

CONCLUSIONS.

1. Regeneration of a thymus transplant is characterized by hypertrophy and hyperplasia of the reticulum cells, leading to the formation of small and large atypical Hassall bodies during the early stages.

2. Regeneration is usually complete by the 3rd week, when the newly formed lobules show differentiation into cortical and medullary zones, and typical Hassall bodies appear.

3. Typical Hassall's corpuscles are also derived from the reticulum epithelial cells.

4. These corpuscles have no function, being aggregates of spent reticulum cells.

5. The thymus reticulum cells are actively phagocytic, and react rapidly when noxious influences are exerted on the gland.

I wish to express my appreciation for the assistance which Miss Alma Adler has given me during the course of this work.

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EXPLANATION OF PLATES.

PLATE 17.

FIG. 1. 4 days. Shows, from left to right, abdominal muscle, connective tissue, peripheral zone of transplant with hyperplastic reticulum cells, and newly formed Hassall's corpuscles. To the right of this zone the agglutinated material of the original transplant is seen.

FIG. 2. 4 days. High power of three Hassall's corpuscles in peripheral zone of Fig. 1. One shows a core of degenerated protoplasmic and nuclear material surrounded by reticulum epithelial cells.

FIG. 3. 10 days. The reticulum epithelial cells show marked hypertrophy, the picture resembling stratified squamous epithelium in places. Hyaline degeneration with the formation of large atypical Hassall's bodies takes place.

FIG 4. 12 days. Shows a regenerated thymic lobule with a central degenerated epithelial core—large atypical Hassall's corpuscle.

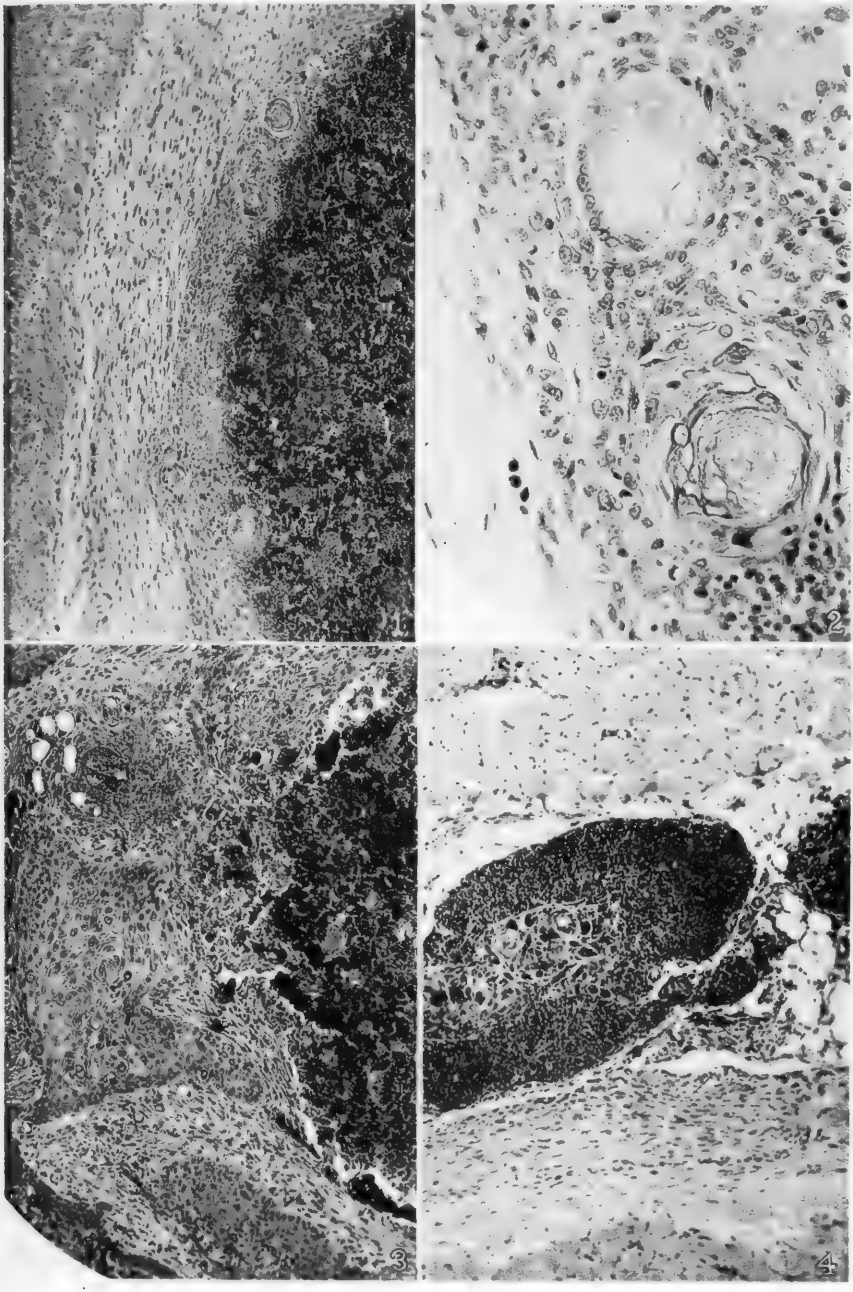
PLATE 18.

FIG 5. 16 days. The central degenerated cores passing from the lobules into the interlobular spaces.

FIG. 6. 30 days. A completely regenerated lobule containing typical small Hassall's corpuscles.

FIG. 7. 30 days. High power of Fig. 6 showing two small typical Hassall's corpuscles.

FIG. 8. 120 days. An involuting transplant with typical small, typical conglomerate, and atypical Hassall's corpuscles. The corpuscle seen near the center is similar to those in the peripheral zone of a 4 day transplant.



(Jaffe: Autoplastic thymus transplants. II.)

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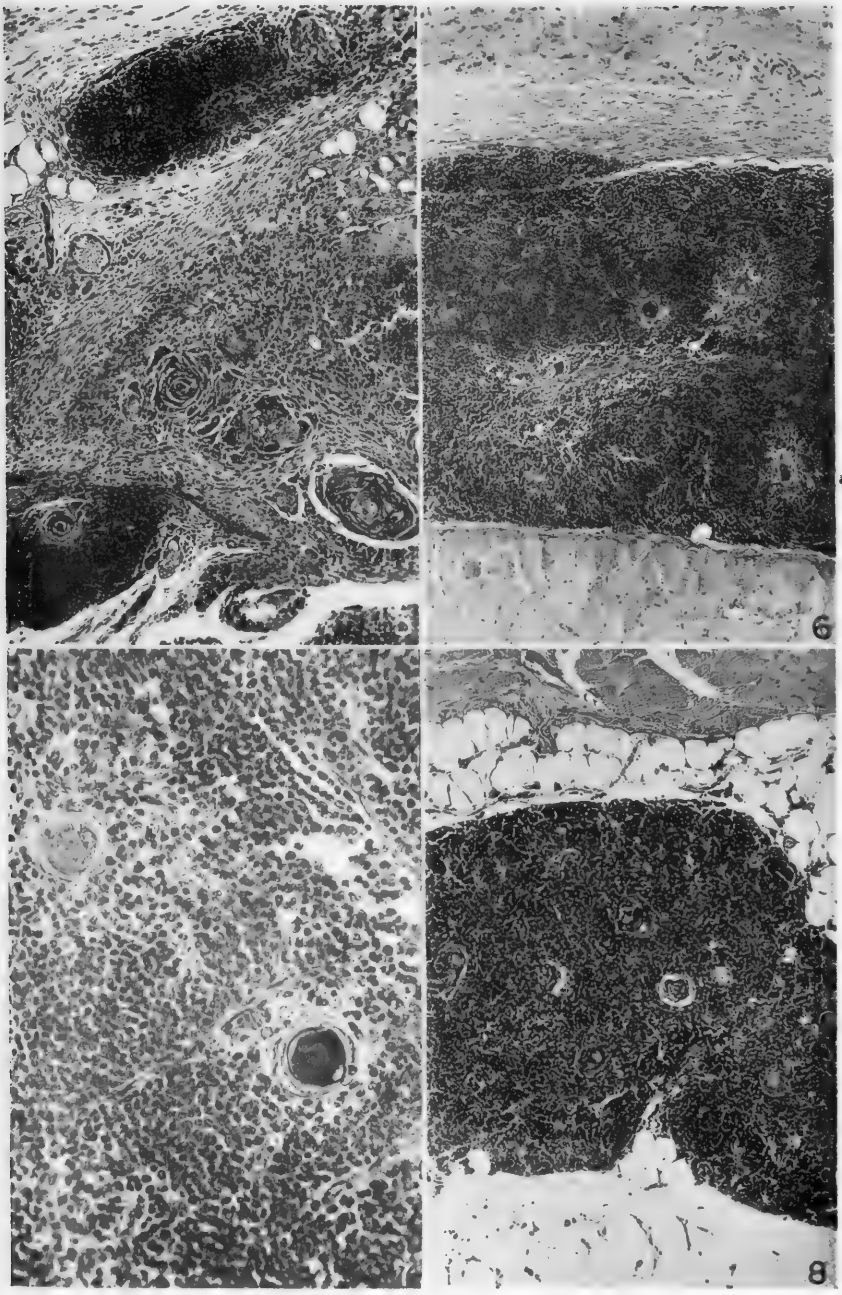

(Jaffe: Autoplastic thymus transplants. II.)

ETIOLOGY OF OROYA FEVER.

II. Viability of Bartonella bacilliformis in Cultures and in the Preserved Blood and an Excised Nodule of Macacus rhesus.

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(Received for publication, June 4, 1926.)

Pure cultures of *Bartonella bacilliformis* were first obtained from a specimen of human blood in citrate solution, transported from Lima, Peru, to New York City in the refrigerator.[1] The first successful cultures were made on October 3, and the second on October 20, 1925, the blood being 28 and 43 days old, respectively, when used for the purpose of cultivation. The fact that pure cultures were obtained on both occasions indicated a surprising ability of *Bartonella bacilliformis* to exist under modified conditions and suggested experiments on the viability of the organism.

Viability of Bartonella bacilliformis in Infective Blood.

Table I shows the results of viability tests made with citrated blood of infected monkeys (*M. rhesus*) kept constantly at 4°C. All of the fifteen specimens, which were derived from seven monkeys infected with cultures of *Bartonella bacilliformis* or monkey passage strains, yielded cultures of the organism when tested after periods of refrigeration varying from 24 to 152 days following withdrawal from the animals. In most instances the number of organisms was considerably reduced, as is evident from the fact that none of the preserved specimens yielded growth in dilutions higher than 1:10, whereas the titer in some instances had been 1:100,000 at the time of withdrawal.

In testing the effect of room temperature upon similar material, two specimens of citrated blood, derived from *M. rhesus* 8[2] and *M.*

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv (in press).

rhesus 12,[3] were employed (Table II). The temperature of the laboratory where the samples of blood were kept varied from 15° to 22°C., and the tall test-tubes serving as containers were stoppered with cotton plugs and were not protected from the light. There was still a trace of what appeared to be hemoglobin in the supernatant portion of the plasma after 45 days, but the general tone had become brownish

TABLE I.

Viability Tests of Bartonella bacilliformis in Citrated Blood Kept at 4°C.

Source of blood	Date of withdrawal	Result of test on Jan. 29, 1926	Days after withdrawal	Result of test on Apr. 12, 1926	Days after withdrawal
Rhesus 1	Nov. 11, 1925	+	79	+	152
" "	Dec. 8, "	+	32	+	104
" 2	" 18, "	+	42	+	114
" "	Jan. 4, 1926	+	25	+	97
" 3	" " "			+	97
" "	" 25, "			+	86
" "	Feb. 6, "			+	74
" 4	Dec. 18, 1925			+	114
" 5	" 22, "			+	110
" "	Jan. 12, 1926			+	89
" 6	Dec. 28, 1925			+	104
" "	Jan. 4, 1926	+	25		
" 7	" " "	+	24		
" 8	Dec. 18, 1925	+	42		

TABLE II.

Viability of Bartonella bacilliformis in Citrated Blood Kept at Room Temperature.

Source of blood	Date of withdrawal	Titer of fresh blood	Result of test on Mar. 20, 1926 (after 45 days)	Titer of blood on Apr. 12, 1926 (after 67 days)
Rhesus 8	Feb. 3, 1926	1:10	+	>1:10,000,000
" 12	" " "	1:10	+	>1:10,000,000

when examination was made 67 days after withdrawal of the blood. As the table shows, the titer of the preserved blood specimens was at least 1:10,000,000, whereas the titer of the fresh blood in each instance had been only 1:10, that is, multiplication had taken place at room temperature. Film preparations made from the specimens showed

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv (in press).

the organisms to be in small clumps scattered among the corpuscles. There was no special tendency to localization within or around the erythrocytes, such as is evident in the blood of human beings suffering from Oroya fever.

Viability of Bartonella bacilliformis in the Nodule from an Animal Experimentally Infected.

Bartonella bacilliformis as it occurs in the local lesions of infected animals is exclusively an intracellular parasite, being found usually in the cytoplasm, occasionally within the nuclei, of proliferating endothelial cells (clasmatocytes). In actively growing nodules the number of microorganisms is very large.

TABLE III.

Viability of Bartonella bacilliformis in the Excised Nodule and Emulsion at 4°C. and at Room Temperature.

Nodule excised on Mar. 1, 1926	Unground tissue		Emulsion	
	4°C.	Room	4°C.	Room
Mar. 7 (7 days).....	+	+	+	+
" 14 (14 ").....	+	+	+	+
" 28 (28 ").....	+	+	+	+
Apr. 4 (35 ").....	+	−	+	−
" 25 (56 ").....	+	−	+	−

The suspension made by triturating with citrate solution a portion of the large subcutaneous nodule that developed on the abdominal wall of *M. rhesus* 14[2] following the injection of a mixture of culture and passage strain yielded a culture of *Bartonella bacilliformis* in a dilution of 1:100,000. This material was used for determining the viability of the organism in the tissue of the excised nodule. The results, which are recorded in Table III, indicate that *Bartonella bacilliformis* survives in an untriturated piece of excised nodule or in the suspension of the tissue for more than 56 days at 4°C. At room temperature, on the other hand, the organisms had died out after a period of 35 days, though growth had been obtained from the suspension after 28 days. The autolysis of tissue taking place at the higher temperature may have been detrimental to the organism, or there may have been

an exhaustion of nutrient substances. It was found, however, that the addition, at the beginning of the experiment, of about 10 per cent of fresh rabbit or horse serum and a trace of hemoglobin to the tubes containing the suspension not only prevented the death of the organism but actually induced growth.

Viability of Bartonella bacilliformis in Cultures.

There are two culture media on which *Bartonella bacilliformis* grows well,[1] the so called leptospira medium and blood agar slants. The hydrogen ion concentration most suitable for growth is pH 7.8 to 8, and it is advisable to adjust the medium to this reaction. It has been shown also, through the cooperation of Dr. J. H. Bauer, that the

TABLE IV.

Viability of Bartonella bacilliformis in Culture on Leptospira Medium.

No. of tube	Temperature	No. of days	Result of transplant	No. of tube	Temperature	No. of days	Result of transplant
	°C.				°C.		
1	25	53	+	9	37	37	+
2	25	57	+	10	37	37	+
3	25	63	+	11	37	47	+
4	25	64	−	12	37	47	−
5	25	75	+	13	37	50	+
6	25	80	+	14	37	60	−
7	25	95	+	15	37	62	−
8	25	120	+	16	37	65	−

substitution of Huntoon's hormone broth or Meyer's peptic digest broth for the ordinary meat infusion broth as the basis of these media has a decidedly favorable influence upon the growth of the organism.

One of the peculiarities of *Bartonella bacilliformis* in culture is that it soon reaches the limit of growth at 37°C., though multiplication continues steadily at 25°C. On leptospira medium the organisms remain viable longer than on blood agar slants, growth progressing for a month or more in the former case, while in the latter the maximum seems to be reached in about 14 days at 25°C., and then only when the evaporation of the medium is retarded by soaking the cotton plugs with paraffin or closing the tubes with rubber stoppers. As a rule *Bartonella bacilliformis* remains motile on blood agar for 10 days, occa-

sionally as long as 14 days, but it is usually non-motile when grown on the semisolid medium. Microscopic examination alone is not sufficient for the detection of viability, therefore; cultural tests must also be made.

Many culture tubes having been preserved from the time of the isolation of *Bartonella bacilliformis* in October, 1925, it was possible to make a series of viability tests, the results of which are recorded in Table IV. In order to retard evaporation, the cultures on leptospira medium intended for preservation were covered with a layer 2 cm. deep of sterile paraffin oil.

As the table shows, a culture of *Bartonella bacilliformis* on leptospira medium at 25°C. remained transferable for a period of at least 120 days, while tubes kept at 37°C. longer than 50 days no longer gave growths on new medium. Culture tubes removed to a refrigerator at 4°C. after their maximum growth had been reached (28 days at 25°C.) were found to be still viable at the end of 4 months.

The viability of the organism on blood agar slants is very inconstant. If the plug closing the tube is not practically impermeable the surface quickly dries, and the organisms cease to multiply and degenerate within a fortnight. On a slant at 25°C. containing sufficient condensation water and closed with a nearly air-tight stopper the organisms remain motile for about 2 weeks and transferable for a month or more. The stopper should not be made absolutely air-tight, because *Bartonella bacilliformis* does not grow in the absence of oxygen.[1] As already stated, a slant culture kept at 37°C. dies out in about 10 days.

SUMMARY.

Fifteen specimens of citrated blood from seven monkeys infected with *Bartonella bacilliformis* were kept at 4°C. for periods of 24 to 152 days, and at the end of each period were tested for viability by the cultural method. All yielded cultures, although there was a considerable reduction in the number of living organisms, as shown by titration.

Two specimens of citrated blood, from infected monkeys, which had been kept for 45 days at room temperature yielded growth when a drop of each was inoculated into leptospira medium; while after 67

days at the same temperature 0.1 cc. of a 1:10,000,000 dilution of each specimen was sufficient to yield growth. Since the original titer of the blood had been only 1:10, it is evident that *Bartonella bacilliformis* had multiplied considerably under the conditions. Smears of the specimens showed clumps of organisms among the corpuscles but no intracorpuscular multiplication.

Bartonella bacilliformis survived in the excised nodule from a monkey for at least 56 days at 4°C., and for 28 days at room temperature, when a piece of the tissue was covered with citrate-saline solution or ground up in it. The death of *Bartonella bacilliformis* at room temperature under these conditions may be due to the effect of autolysis of the tissue or to a lack of nutrient substances. The suspension alone is not a suitable culture medium, but a trace of hemoglobin and about 10 per cent of fresh horse or rabbit serum make it a favorable one.

The viability of *Bartonella bacilliformis* was tested in cultures kept at 25°, 37°, and 4°C. for varying periods. At 25°C. cultures on leptospira medium remained transferable after 120 days, and when placed in the refrigerator at the time of maximum growth (after 28 days at 25°C.) they were still viable at the end of 4 months. The viability of cultures on blood agar slants depends to a considerable extent upon the care with which the surface of the medium is protected from drying. Under favorable conditions and at 25°C. the organisms remain motile for about 2 weeks and transferable for a month or longer. Cultures on either of these media die out after 50 days at 37°C.

The data presented suggest that it may be fruitful to make a study by cultural methods of pathological material brought from distant parts of the world, even when many days or weeks have elapsed since it was procured.

THE RELATION OF BACTERIUM PNEUMOSINTES TO INFLUENZA: A STUDY WITH A STRAIN OF THE ORGANISM DERIVED FROM THE NASOPHARYNGEAL WASHINGS OF A CASE OF INFLUENZA.*

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The laborious and carefully planned researches of Olitsky and Gates on the etiology of influenza, initiated during the great outbreak of 1918, and continued to the present time (1923) through several epidemic recurrences, have opened up an entirely new field in the investigation of this disease and of others of as yet unknown causation. The details of their findings are too well known to require detailed recapitulation. Suffice it to say that they have isolated from patients in repeated outbreaks of the disease, a minute, anaerobic organism, cultivable with difficulty, which when injected intratracheally into rabbits produces lesions which they believe to be specific and which resemble the pulmonary lesions of influenza as well as these can be deduced from the large amount of autopsy material on record. Animals infected with this organism (intratracheally) have, in their hands, proven susceptible to pulmonary localization of concurrently present microorganisms of the common respiratory types, with the production of typical pneumonias, whereas the same organisms, in animals without *Bacterium pneumosintes*, if producing infection at all, fail to localize in the lungs and cause instead general septicemias. The clinical symptoms produced by *Bact. pneumosintes* alone are very inconspicuous, consisting in a rise in temperature, combined with a leucopenia believed by them to parallel that of influenza[1] and occasionally conjunctivitis, but without other evidence of illness on the part of the animal.

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[1] The leucopenia observed in rabbits by Olitsky and Gates after *pneumosintes* inoculation is characterized by a relatively great reduction in the number of lymphocytes. Most reports on influenza describe the leucopenia in that disease as showing a relative reduction of the polynuclear neutrophils and increase in the proportion of lymphocytes.

This action of the organism on the rabbit accords well with the conception of influenza current at the present time which presents this disease as a mild illness of short duration, characterized by fever, leucopenia, inflammation of the upper respiratory tract, and a marked predisposition to secondary pulmonary infection by organisms of the types usually found in non-influenzal inflammations of the bronchi and lungs. From the standpoint of serious disease and mortality, therefore, the disease may be conveniently regarded as an infectious predisposition to pneumonia. This is apparently exactly the action of *Bact. pneumosintes* upon the rabbit, and taking into consideration the probability of this animal being not as susceptible to the action of this virus as others may be, notably man, the parallelism between their experimentally induced condition, and the natural disease in man, appears sufficiently striking. The probability of the relationship to influenza of *Bact. pneumosintes* is increased by the results of agglutination tests on the blood of convalescents performed by Olitsky and Gates (1921–22) in recent outbreaks which have given apparently specific results.

The general attitude of the profession with regard to this work, however, remains one of skepticism, and general confirmation of their findings is necessary before the organism of Olitsky and Gates can be accepted as the specific cause of influenza. This attitude on the part of the profession is perhaps partly due to the reaction from the too great ease with which the conclusions of Pfeiffer were accepted a generation ago, and the desire to be more than sure that a similar error is not committed at this time. Confirmation of their findings has been attempted by many, a few of whom have published their results. At least partial confirmation has been had at the hands of Loewe and Zeman (1920), who have isolated an organism apparently identical to *Bact. pneumosintes* from influenzal cases. Gordon (1922), Lister (1922), and Detweiler and Hodge (1924) have also partially confirmed this work. Others have failed in similar attempts. Certain details of the work have been criticized by several writers, notably the production of characteristic lesions in the rabbit (Maitland, Cowan, and Detweiler, 1920). Owing to the time-consuming and technically difficult procedures involved in the isolation of this organism, and the fact that only at times of epidemic outbreaks is undoubted influenzal

material available for experimentation, it is perhaps not to be wondered at that confirmation, even if ultimately forthcoming, should be slow in appearing. The attempt to repeat the work of Olitsky and Gates in its entirety is manifestly beyond the resources of most laboratories, and it therefore seems justified for the individual investigator to take up special features of their work and such fragmentary observations as are secured in this way should be placed on record to the end that a final decision as to the standing of *Bact. pneumosintes* may be reached as soon as possible. It is the purpose of the present report to record a study of the clinical effects and pathological lesions observed in experimental animals after inoculation with influenzal material, and the results of some attempts to cultivate the organism.

During February, 1922, an epidemic of acute respiratory disease occurred in the city of Washington. It was clinically typical influenza of a type in general greatly milder than that observed in 1918, but was accompanied by a very distinct increase in the incidence and mortality of pneumonia in the city at large. At the neighboring military post of Fort Myer, the epidemic, while unaccompanied by any fatality, ran a characteristically explosive course which, with the equally characteristic clinical characters of the disease, left no doubt in the minds of observers as to the nature of the outbreak. From one of the cases of this epidemic samples of blood and of nasopharyngeal washings were taken February 17, 1922. This soldier was at the time within 24 hours of the onset of the disease. He complained of a sudden onset, severe headache mainly postocular, sore throat, hoarseness, marked pain in bones and muscles, and great prostration, this last persisting for several days after defervescence. His temperature at the time the material was taken was 102.6°F., and his blood contained 5500 white cells per c.mm., of which only 47 per cent were polynuclears. His temperature became normal on the 2nd day following and except for the weakness which persisted, his convalescence was uneventful. Culture of the blood under aerobic conditions was reported as negative, while cultures from throat swabs and of the nasopharyngeal washings showed the presence of the Pfeiffer bacillus and of *Streptococcus viridans*. Inoculation of a mouse intraperitoneally with the nasopharyngeal washings resulted in the isolation of a pneumococcus of Group IV.

EXPERIMENTAL WORK.

The nasopharyngeal washings from this case were shaken with sterile glass beads to a uniform emulsion, and the resulting turbid fluid, which had about the opacity of the Army triple vaccine was used for animal inoculations. One rabbit and two guinea pigs were inoculated intratracheally, of which the two latter promptly died.[2] One guinea pig was inoculated with the unfiltered washings subcutaneously on account of the writer's earlier results with influenzal filtrates injected subcutaneously and intravenously (Hall, 1920). A rabbit was also inoculated with the citrated plasma from the blood of the patient. The results in these experiments will be recapitulated briefly.

First Animal Passage.

The rabbit inoculated with the citrated blood plasma from the patient showed a slight elevation of temperature on the 2nd day after inoculation but no leucocyte reaction. It was not killed until the 5th day. The lungs appeared normal in the gross but on microscopic examination showed a diffuse edematous thickening of the alveolar walls. The results, while perhaps suggestive, seemed so doubtful that no further work was done with material from this animal.

Rabbit 1 received intratracheally 3 cc. of the unfiltered nasopharyngeal washings from the case described. On the 2nd day there was a rise of temperature (1°F.), and a drop of 25 per cent in the total leucocyte count and of 40 per cent in the number of mononuclear leucocytes. The animal was killed and examination of the lungs showed a well marked diffuse lesion of the type discussed later. There was no microscopic evidence of pyogenic infection although cultures from lung and trachea were positive for *B. lepisepticus*. Portions of the lung emulsion from this rabbit were carried on in two rabbits and one guinea pig.

Guinea Pig 1 received 2 cc. of the unfiltered nasopharyngeal washings subcutaneously. It showed no temperature reaction. On the 4th day there was a definite drop in the total leucocyte count (40 per cent) and a somewhat greater fall in the mononuclears. The animal was sacrificed and microscopic examination of the lung showed the same diffuse lesion observed in Rabbit 1. In addition there were areas of the chronic proliferative lesion described by Maitland and so commonly seen in guinea pigs. The lung of this animal was preserved for several days in 50 per cent glycerol in the ice box and then emulsified and injected into Guinea Pigs 2 and 3. Cultures of fresh and glycerolated lung tissue (chocolate agar) remained sterile.

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[2] Olitsky and Gates gave up the use of guinea pigs on account of the difficulty of successful intratracheal inoculation with this material, and on account of the great frequency of concurrent pulmonary infections in these animals. All intratracheal inoculations were done by operative exposure of the trachea under ether anesthesia, the injection being done with a fine curved needle.

Second Animal Passage.

Rabbit 2 was inoculated intratracheally with unfiltered material from Rabbit 1. This material contained *B. lepisepticus*. On the day following inoculation the animal showed a rise of temperature which continued until the 3rd day (104.4°F.), when it was sacrificed. It showed during this time a progressive fall in total leucocytes and mononuclears. Microscopically the lung showed a fairly well marked diffuse lesion of the same type as did Rabbit 1, with scattered foci of suppurative pneumonia and areas of old proliferative change.

Rabbit 3 received 3 cc. of the same material as the one last described but filtered (Mandler) and aerobically sterile. There was no thermic or leucocytic reaction. There was no diffuse pulmonary lesion and the experiment was regarded as definitely negative.

Guinea Pig 4 received subcutaneously 3 cc. of the unfiltered lung emulsion from Rabbit 1 which contained *B. lepisepticus*. There was no thermic or leucocytic reaction and examination of the lung showed no diffuse pulmonary lesion.

Guinea Pig 2 was injected intratracheally with 0.5 cc. of lung emulsion from Guinea Pig 1, unfiltered but aerobically sterile. There was a definite progressive rise in temperature, no fall in total leucocytes, but a decided absolute reduction in mononuclears. The animal was sick with rapid respiration, palpable râles in chest, and cough. Sacrificed on the 2nd day after inoculation. The microscopic findings were those of an acute suppurative bronchopneumonia. There was no evidence of the diffuse lesion seen in the earlier animals. However, the prompt production of a pneumonia without leucocytosis by the introduction of material sterile to ordinary cultural test is suggestive of the action of *Bact. pneumosintes* as described by its discoverers.

Guinea Pig 3 received subcutaneously 2 cc. of the same material as the above. This animal showed a slight rise in temperature and a marked fall in leucocytes, especially the mononuclears. It showed, when sacrificed on the 3rd day following inoculation, a well marked diffuse lesion of the type to be described as characterizing *pneumosintes* animals.

Of these eight animals, one may be thrown out as having received blood plasma rather than nasopharyngeal material. Of the remaining seven which received the latter either in the first or second passage, Rabbit 1 showed a typical *pneumosintes* reaction of fever, and fall of leucocytes after 48 hours. Guinea Pig 1, after 4 days incubation (inoculation was subcutaneous) showed a marked leucocytic reaction but no fever. Of the five animals receiving material in the second passage, Rabbit 2 showed fever and leucocyte drop after 3 days; Rabbit 3 receiving filtered material gave an entirely negative result; Guinea Pig 4 showed a slight rise of temperature after 48 hours but no

leucocyte reduction. These three all received material from Rabbit 1 which had given a satisfactorily positive reaction to primary inoculation. The two guinea pigs inoculated with material from Pig 1 both showed decided reactions; No. 2 developed fever with sharp drop in mononuclear leucocytes, the total count remaining the same. This pig showed a definite pneumonia due to the organism so commonly found in guinea pig and rabbit lesions, *B. lepisepticus*. Pig 3 injected subcutaneously gave a very typical leucocyte curve and a slight rise in temperature. Of the seven animals then, four showed to some degree the *pneumosintes* reaction as defined by Olitsky and Gates, one other showed a doubtful reaction which, however, may be considered positive inasmuch as it was material from this animal which was used in the inoculation of Pig 3, which gave a definitely positive reaction, and from which, as will be shown later, there was isolated an organism which has been identified as *Bact. pneumosintes*. Two of the animals gave negative results. Without going into more detail with regard to individual animals, it may be said that further passage of the virus resulted in practically the same way, a certain number of doubtful or apparently negative reactions occurring in each series of inoculations.

In the discussion of the Rockefeller Institute studies much has been said as to the specificity of the pulmonary lesions described by Olitsky and Gates as occurring in rabbits inoculated with influenzal material and not in control animals. Their original description of the pulmonary findings in rabbits infected intratracheally with *pneumosintes* material, which so far as I know has not been since amplified, is as follows:

"*Pathological Effects.*—The respiratory organs were affected to the exclusion of all others. No pleuritis or exudate in the pleural cavity was evident. The lungs were voluminous as a result of edema and emphysema and had a mottled hemorrhagic appearance. The hemorrhages on the surface, beneath the pleura, were diffuse or discrete, occupying areas a few millimeters in extent or covering a large part of a lobe. In addition, minute petechiæ were seen scattered over the entire surface. On section of the lungs the cut surface revealed a hemorrhagic edema; it dripped a blood-stained, frothy fluid. The hemorrhages again were either diffuse and large, or discrete and small, in the latter instance being numerous.

"On microscopic section carried through various parts of the lungs the lesions were found to consist (*a*) of hemorrhagic foci, and (*b*) of edema and emphysema. The hemorrhages varied in size in accordance with the observed macroscopic

appearance, some being microscopic in nature. The edema was more extensive than the hemorrhages and involved alveoli and interalveolar strands of tissue. The alveoli contained coagulated serum or red corpuscles, mononuclear cells, and also at times polymorphonuclear cells of eosinophilic type and desquamated epithelial cells. The interalveolar strands were infiltrated with mononuclear cells and large cells the foreign nature of which was not always clear. Fibrin was sometimes present in small amounts. The bronchi, also, were at times filled with erythrocytes, exfoliated and degenerated epithelia, and leucocytes. The capillaries were distended with blood."

The authors do not mention any variations in the pathologic picture encountered in their experimental animals, such as would be expected to occur in any series of inoculations with a given virus, nor have they indicated that the lesions produced by *Bact. pneumosintes* isolated in later outbreaks of influenza produced less marked changes than those described. Certain it is that in degree, at any rate, the changes shown in the lungs of the animals that I have indicated as probably positive in the series under discussion do not correspond to the above description. The appearance of the lung on removal seldom corresponded to the description given. Only exceptionally was the voluminous appearance observed. Red or hemorrhagic spots on the surface occurred in all the animals of the series, but these spots varied so greatly in color, shape, and size that no description can be given that can be regarded as characteristic. Moreover, such spots are commonly found in control animals. The moist bloody condition of the section occurred regularly in my series, varying considerably in degree. The microscopic picture of my positive cases corresponded better with the photomicrographs presented by Olitsky and Gates than with their written description. The outstanding feature impressing one at first glance was in every case a *diffuse* thickening of the interalveolar walls. On examination with the 4 mm. objective, this thickening was seen to be due primarily to edema, separating the epithelial linings of adjacent alveoli. The capillaries in the walls were engorged and in some places interstitial hemorrhages were evident. Evidence of alteration of the blood in hemorrhagic areas is often seen. Infiltration by cells of a lymphoid type and also by larger cells resembling endothelium was constant though varying in degree. In the rabbits, scattered eosinophil polynuclears were usually seen. Intra-alveolar edema occurred in all cases but was usually of very limited

extent. Cellular exudation into the alveoli was not observed in this series, nor was hemorrhage or exudate observed in the bronchi. In other words, the findings in this series of cases might well correspond to those produced by an agent of a character similar to that of Olitsky and Gates, but acting with much less intensity, provided always that control animals fail to show the same changes.

Control Experiments.

It is necessary before drawing conclusions as to the presence or absence of a distinctive lesion in the animals studied above to establish the fact that such changes in the lungs as have been observed are the result neither of the method used for killing the animal, nor of the intratracheal injection of non-infectious material. The latter possibility is ruled out if, as has been the case in my series, characteristic pulmonary lesions may be produced by other than intratracheal routes of injection. I have, moreover, in connection with other work, given many intratracheal injections with sterile lung emulsions, filtered and unfiltered, without observing the lesion described above. For these reasons I will confine my attention at this time to a study of the effects upon the conditions observed in the lungs after various methods of killing experimental animals. It would appear too far fetched to assume that a hemorrhagic, edematous, and exudative lesion could occur in normal animals.

Some twenty-five rabbits, and a smaller number of guinea pigs, were studied in the attempt to establish satisfactory control conditions. Animals were killed by the inhalation of chloroform and ether, by subcutaneous injection of potassium cyanide, by pithing, and by the intracardiac injection of a saturated solution of magnesium sulfate. None of these methods produced with uniformity lungs as nearly normal on gross and microscopic examination as the method used by Olitsky and Gates, that of dislocating the cervical spine by a single properly directed blow. This method will occasionally fail to produce immediate death, though paralysis and apparent loss of consciousness result immediately. When this occurs agonal lesions in the lungs are observed. Careful microscopic study of a considerable series of normal animals killed in this way leads to the conclusion that there are definite differences between the lesions thus produced and those found in the positive cases of the influenzal series. It is true that areas of hemorrhage are often observed in these control cases, but in no case has the lesion presented the diffuse distribution found in the influenzal series. The hemorrhage in the control cases is often of interstitial character, indeed usually is so, but the evidence of interstitial

edema is entirely wanting. Furthermore, the cellular infiltration of the alveolar walls is absent. The hemorrhage is evidently very recent in point of time and no evidences of alteration are seen in the red cells. Such alterations are frequently observed in the influenzal series. (The presence of large pigmented cells is common in all rabbits as also are the eosinophilic leucocytes.) Animals killed by chloroform or ether give a microscopic picture more nearly approximating that of the influenza series, while those killed by intracardiac injection of magnesium sulfate show in most cases an intense engorgement of the pulmonary capillaries, with occasional hemorrhage into the alveoli.

The conclusion to which we are led by these observations is that the lung of a rabbit or guinea pig is very easily injured with the production of hemorrhagic lesions. That method of killing which produces most nearly instantaneous death without struggle or muscular spasm on the part of the animal seems to show on examination the most nearly unaltered lung tissue. Methods of killing somewhat less than instantaneous will give a greater or lesser proportion of hemorrhagic lesions on examination, the least extensive of these lesions showing an interstitial distribution, confined to the interalveolar walls. Such lesions are never diffuse and are characterized by the presence in the walls of fresh unaltered blood without edema or other evidence of inflammatory reaction in the form of cellular infiltration.

As a test of the validity of these perhaps slight distinctions in the pathology of these lungs I submitted a series of slides to Major George R. Callender, of the Army Medical Museum. These included two from rabbits with the influenzal lesion, one killed by chloroform, one by magnesium sulfate injection into the heart, and one by a blow. He promptly identified the two influenzal slides as an acute inflammatory process of similar nature in the two cases, the one from the animal killed by a blow as practically normal in spite of several localized purely hemorrhagic lesions, the one killed by magnesium sulfate as a passive congestion, while the chloroform case was the cause of some hesitation, but finally was placed as a congestion and edema without evidence of inflammatory reaction. My conclusion that the lesion seen in the influenzal cases is specific is also fortified by a previous observation (Hall, 1920) in which a rabbit inoculated intravenously with the filtered lung substance of another similarly injected with the filtrate of the sputum from an early case of influenza, died at the end of 40 hours.

Careful examination of this animal anatomically, histologically, and bacteriologically failed to show any cause of death other than an extreme hemorrhagic and edematous lesion of the type described for the influenza animals. The entire lung of this animal was of a red meaty appearance and the elasticity of the lungs must have been entirely destroyed. The animal had exhibited great difficulty in breathing and apparently died of suffocation. So far as I know this is the only rabbit which has succumbed to the uncomplicated action of a virus that could reasonably be interpreted as influenzal. The occasional occurrence of animals unusually susceptible to the action of this virus is perhaps to be expected from the similar occasional occurrence of fulminant cases in man.

In the light of the foregoing considerations, it appears to me justifiable to assume that the series of animals in question had received an agent which is capable of inducing a pathological condition in rabbits and guinea pigs characterized, after an incubation period of 1 or 2 days, by some elevation of temperature and reduction of the leucocytes, especially the mononuclears, and, when killed at this time, also showing a pulmonary lesion which is distinguishable from those accidentally incurred at time of death from other causes.

Cultivation Experiments.

When the opportunity for study of this case arose there was available no suitable ascitic fluid for the preparation of the Smith-Noguchi medium. Tubes satisfactorily controlled for sterility were first available at the time of the second animal passage and attempts at cultivation were made with material from all of the animals of this group. Of these only Guinea Pig 3 gave positive results. This animal had been inoculated subcutaneously with the unfiltered lung emulsion from Guinea Pig 1 which had proven sterile on aerobic cultivation on chocolate agar.

The turbidity of the inoculated tubes became noticeably greater than that in controls after about 10 days at 37°. Microscopic examination of these tubes showed minute coccobacillary forms, Gram-negative, and corresponding to the descriptions of *Bact. pneumosintes* as given by Olitsky and Gates. Subcultures on the same medium resulted in continuation of the growth and after three transfers it became possible to secure a good growth and evident colony formation on blood agar plates incubated in the Brown modification of the McIntosh and Fildes anaerobic jar. Emulsions of this growth readily

agglutinated in low dilutions with serum prepared against *Bact. pneumosintes* and kindly sent me by Dr. Gates, while failing to agglutinate with normal rabbit serum. Once adapted to growth on blood agar the strain was readily maintained by weekly transplants. Injected into animals this organism produced the same reactions as already described for the nasopharyngeal washings of the patient and for the emulsions of lung tissue. The relatively long sojourn in artificial media resulted in considerable diminution of the activity of the organism so that the proportion of animals injected which could be considered as giving positive reactions was smaller than was the case with the original material but sufficient, I believe, to show the connection between it and the lesions and clinical reactions. I was never able, however, to recover the organism from the lungs of animals so inoculated.

Lung-Injuring Properties.

One of the most telling points in the recorded work of Olitsky and Gates, tending to indicate that the action of *Bact. pneumosintes* on animals corresponds to that of the influenza virus on man, is the property of the organism to which it owes its name, that of so injuring the lung as to predispose an infected animal to pulmonary inflammations. These workers found that the guinea pig was an unsuitable animal for experimental work with *Bact. pneumosintes* on account of the frequency of resultant secondary infection of the lungs by organisms commonly found in the respiratory tracts of these animals. Such secondary infection was more rare in rabbits. However, concurrent infections by common bacteria of the respiratory tract were reported as readily induced experimentally.

If it can be conclusively shown that animals infected with *Bact. pneumosintes* are rendered seriously ill or killed by doses of various secondarily infecting organisms so small that control animals show little if any ill effect from their administration, an interesting point in the chain of evidence connecting *Bact. pneumosintes* with influenza will be made. The common organisms found in the respiratory diseases of man, the pneumococcus, for instance, when injected intravenously, show no tendency to localize in the lungs of experimental animals. When death is induced by large doses of a virulent strain

it is with the production of a septicemia without pulmonary localization. For this reason experiments were planned for the purpose of testing this point. There follows the description of one such experiment.

Three rabbits were placed under observation for several days, daily records being made of temperature, total leucocyte count, and differential count. On a given day two of these animals (Nos. 4 and 5) were injected intratracheally with 2.5 cc. of the unfiltered lung emulsion of Rabbit 6, of the series inoculated with the pure culture of *Bact. pneumosintes* isolated from Guinea Pig 3. Both these animals showed on the following day a drop in the leucocyte count as compared to previous records, which mainly involved the mononuclear elements, and one of them (No. 5) showed a slight rise in temperature. The latter, together with the third animal (No. 7) which had received no *pneumosintes* material, received intravenously 4 cc. of an 18 hour culture of Pneumococcus Type I, whose virulence had previously been shown to be insufficient to kill at that dose. The latter animal promptly developed a sharp rise in temperature with a doubling of the leucocyte count which persisted for several days with ultimate decided improvement. The animal was killed and the lungs and blood cultured for pneumococci, and the lungs examined microscopically. The latter showed no significant variation from normal and pneumococci were not isolated. The *pneumosintes* animal which received the pneumococcus also showed on the following day a sharp rise in temperature, 3°F. above previous records, but the leucocyte count remained practically constant until the 4th day when there was a drop to 3600, with a temperature of 106.4°F., and the animal died shortly thereafter. Pneumococci of Type I were recovered from the lungs and blood of this rabbit, and microscopic examination of the lungs showed, in addition to the usual signs of *pneumosintes* infection, diffuse invasion of the interalveolar walls by polynuclear leucocytes, with here and there the formation of minute abscesses, and a small amount of exudation into the alveoli. The rabbit receiving *pneumosintes* alone made the usual uneventful recovery.

This and several other similar experiments appear to bear out the contention of Olitsky and Gates that *pneumosintes* material predisposes animals to pulmonary invasion by organisms which ordinarily show no such tendency.

No attempts were made to determine the filterability of the strain of *pneumosintes* recovered at this time as it appears that this property of the organism has no direct bearing on the question of its relation to influenza, and the time at our disposal was limited. No further cases presented themselves for study owing to the prompt subsidence of the epidemic. No extensive control work on normal individuals has been attempted.

SUMMARY.

Nasopharyngeal washings from a case of epidemic influenza have proven capable of initiating a pathological change in rabbits and in guinea pigs characterized after an incubation period of 1 or 2 days, by some elevation of temperature, reduction in the number of circulating leucocytes, especially of the mononuclears, and by a pulmonary lesion during the period of reaction, which is distinguishable from those accidentally incurred at the time of death.

From one such animal, in the second passage of the virus, an anaerobic coccobacillus, corresponding in all respects to *Bact. pneumosintes*, was isolated by the method employed by Olitsky and Gates.

This organism also proved capable of initiating the pathological change in animals found after inoculation with influenzal material.

The observation of Olitsky and Gates that the presence of this organism in the lungs of experimental animals predisposes to pulmonary localization of other bacteria with the production of definite pneumonic lesions has been confirmed.

Bact. pneumosintes infections may be induced by subcutaneous injection of infected material.

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THE NATURE OF THE TOXIN-ANTITOXIN FLOCCULATION PHENOMENON.

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Botulinus antitoxin is a specific remedial agent of experimentally demonstrable therapeutic value. However, in clinical cases of botulism the absence of any reliable criteria for early diagnosis precludes the timely application of the serum therapy. The clinical diagnosis of *botulinus* poisoning is made only from those central nervous symptoms which by their very presence foretell the probable fatal termination of the disease.

In an earlier series of experiments it was found that ingested *botulinus* toxin may be detected in the blood of experimental animals before the onset of unmistakable symptoms of poisoning.[1] The blood of larger animals to which toxin had been fed was injected into mice. The presence of the toxin could be established only after its concentration in the blood of the donor animals had reached a comparatively high level, so that the amount injected into a mouse would contain at least one minimal lethal dose. But the recent publications of Ramon would seem to indicate that it may be possible to detect lower concentrations of toxin by means of precipitation with a specific antitoxin (1–3).

With the problem of early diagnosis of botulism in view, we set out to study the mechanism of the Ramon test. Attempts were made to precipitate 4 day toxin by homologous antitoxin. Ramon had shown that when a series of varying amounts of diphtheria toxin and antitoxin are combined and incubated at an appropriate temperature, there appears a flocculent precipitate in a zone of dilutions which corresponds roughly with those mixtures that are neutral in guinea pig tests.

[1] Bronfenbrenner, J., and Weiss, H., unpublished results, 1922.

Having previously determined that our strain of *B. botulinus* yields its most active toxin in the filtrates of 4 day cultures (4), we prepared such filtrates, and found them to contain approximately 100,000 guinea pig M.L.D. per cc. When incubated for a week with the addition of 0.7 per cent formalin, this so called anatoxin (5), was found to be devoid of all toxicity. The rabbits immunized with the anatoxin yielded sera of high antitoxic value. When pooled rabbit serum was titrated in mice, 0.1 cc. protected the animals against approximately 6000 fatal doses of toxin. Precipitation tests were made with a constant amount

Protocol I.

Flocculation of Pooled Antitoxic Serum by Toxic Filtrates of B. botulinus.

	Amount of antigen	.02 cc. of pooled antitoxic serum			Amount of antigen	.02 cc. of pooled antitoxic serum	
		Filtrate of 3 day culture M.L.D. = .00001	Filtrate of 16 day culture M.L.D. = .001			Filtrate of 3 day culture M.L.D. = .00001	Filtrate of 16 day culture M.L.D. = .001
1	.8	—	—	15	.001	—	—
2	.6	—	—	16	.0008	—	—
3	.4	—	—	17	.0006	—	—
4	.2	—	—	18	.0004	—	—
5	.1	—	—	19	.0002	—	—
6	.08	—	—	20	.0001	—	—
7	.06	—	—	21	.00008	—	—
8	.04	—	—	22	.00006	—	—
9	.02	—	—	23	.00004	—	—
10	.01	—	—	24	.00002	—	—
11	.008	—	—	25	.00001	—	—
12	.006	—	—	26	Control of antigen	—	—
13	.004	—	—	27	" " serum	—	—
14	.002	—	—				

of the serum and a series of dilutions of antigen (4 day toxin) so that the zone of precipitation, if narrow, might not be missed. Toxins of various ages and activity were tested, and the tests were repeated a number of times. All were negative, as shown in Protocol I.

Suspecting some error in technique, we made parallel tests with diphtheria toxin and antitoxin obtained from the New York City Department of Health. The typical flocculation of these mixtures followed, as shown in Protocol II, but the parallel series with *botulinus* toxin-antitoxin remained clear. We varied the hydrogen ion con-

centration, the concentration of electrolytes, and the degree of dilution, but no specific precipitation resulted within the limits of changes not affecting the serum controls. One sample of *botulinus* antitoxin received from the Department of Health gave a very weak and irregular precipitation, but only when this serum was combined with the filtrate of a 24 day culture.

Protocol II.

*Flocculation of Diphtheria and Botulinus Toxins with Their Respective Antitoxins.**

		Diphtheria toxin		*Botulinus* toxin	
		.5 cc. of a 4 L+ toxin	.2 cc. of a 11 L+ toxin	.25 cc. of 4 day filtrate	.25 cc. of 16 day filtrate
Amount of homologous antitoxin	.3000	−	−	−	−
	.2000	−	−	−	−
	.1500	−	−	− (N.)	−
	.1000	−	−	−	−
	.0750	−	−	−	−
	.0500	−	−	−	−
	.0300	−	−	−	−
	.0200	−	−	−	−
	.0150	−	−	−	− (N.)
	.0100	<+	−	−	−
	.0075	<+	<+	−	−
	.0050	++ (N.)	+ (N.)	−	−
	.0030	++	++	−	−
	.0020	−	++	−	−
	.0015	−	+	−	−
	.0010	−	<+	−	−
	Serum control	−	−	−	−
	Antigen "	−	−	−	−

(N.) is the calculated neutral point.

* In this, as well as in other experiments, ++ = copious precipitation; +, fair amount of precipitation; <+, slight amount of precipitation; −, no precipitation.

Having previously observed (6, 7) that precipitation occurs when *botulinus* antitoxin (obtained from the New York City Department of Health) is combined with bacterial autolysates or with extracts of foods infected with *B. botulinus*, we suspected that the present failure might be attributed to the purity of our antigen, since a filtrate of a 4 day culture could be presumed to contain relatively a low concen-

tration of bacterial protein. The precipitate in Ramon's tests might result, we thought, not from actual antitoxin content of the serum, but from the presence in the serum of an antibacterial antibody. In order to test this notion, we prepared an antiserum which, in addition to antitoxin, might contain antibacterial antibody.

Protocol III.

The Influence of Antibacterial Antibody upon the Flocculation of Antitoxin by Toxins.

Amount of antigen (filtrate)	.05 cc. of antiserum to the 4 day toxic filtrate (no antibacterial antibody).		.05 cc. of antiserum to the 24 day toxic filtrate (antibacterial antibody present)	
	Toxic filtrate of 4 day culture 100,000 M.L.D./cc.	Toxic filtrate of 16 day culture 100 M.L.D./cc.	Toxic filtrate of 4 day culture 100,000 M.L.D. per cc.	Toxic filtrate of 24 day culture 100 M.L.D. per cc.
cc.				
1.000	–	–	–	–
.750	–	–	–	–
.500	–	–	–	–
.300	–	–	–	–
.200	–	–	–	–
.150	–	–	–	<+
.100	–	–	–	+
.075	–	–	–	+
.050	–	–	–	+
.030	–	–	<+	++
.020	–	–	+	+
.015	–	–	<+	<+
.010	–	–	<+	<+
Control of antigen	–	–	–	–
" " serum	–	–	–	–

Immunization with Filtrates of Old Cultures.

For this purpose, we immunized a new set of animals with the filtrates of 24 day cultures of *B. botulinus*. Although the filtrates contained only 100 guinea pig M.L.D. of toxin per cc., as determined by preliminary titration, they were assumed to contain the products of bacterial autolysis.

The filtrates were incubated as previously with 0.7 per cent formalin for a week, and then employed for subcutaneous injection in rabbits. When the pooled serum obtained from these animals was tested against the filtrate of the 24 day

culture or against the anatoxin prepared from it, the typical flocculation zone was brought out without difficulty. However, when it was tested against the filtrates of the 4 day cultures which we had used earlier (Protocol I), the second serum gave a definite flocculus, but it was less in amount and restricted to a narrower zone (Protocol III) (8).

The best results were obtained when the ingredients were measured directly, with a micro pipette, and without dilution. The racks were placed in the water bath at 55°C. for 20 minutes, in the 37° incubator for 18 hours, and then in the ice box for 24 hours. Readings were made at the end of each interval, and finally the flocculus was stained and examined for the presence of bacterial growth.

Immunization with Washed Bacteria.

Preceding experiments having shown that an antiserum containing antitoxic antibodies alone will not cause precipitation of a toxic filtrate, but that an antiserum that contains antibacterial antibody in addition to the antitoxin will cause such precipitation, it seemed worth while to determine whether an antibacterial serum wholly devoid of antitoxin would cause precipitation of a toxic filtrate.

A 24 hour culture of a rapidly growing strain of *B. botulinus* was filtered from a beef heart medium through a paper filter to remove the meat particles. The cloudy filtrate was centrifuged and the sediment of bacteria resuspended in saline, and then centrifuged again. Washing was repeated a sufficient number of times to insure the elimination of toxin by dilution. The final suspension of bacteria in saline was then shaken with glass beads with a few drops of chloroform in a shaking machine for 48 hours and filtered. The final filtrate up to 1.0 cc. of autolyzed bacteria was found non-toxic by intraperitoneal injection in mice. This material was used for the subcutaneous immunization of rabbits.

The serum thus obtained did not contain enough antitoxin in 0.1 cc. to protect a mouse against even a single M.L.D. of a Type A toxin. Yet the serum, when combined with the toxic filtrates of cultures of various ages, gave in each case definite precipitations in a comparatively wide zone. It yielded similar reactions in approximately the same zone with anatoxins made from toxins that had originally contained one-hundredth the number of M.L.D. per cc. (Protocol IV). As there was no antitoxin in the system, the precipitation could not have been influenced by antitoxic antibodies.

Immunization with Filtrates of an Atoxic Variant.

The experiments described above suggest that the precipitation of toxic filtrates of *B. botulinus* may be wholly independent of the antitoxin content of the precipitating serum. In order to determine this point, an antiserum was prepared by the immunization of animals with a homologous atoxic strain of the bacillus.

Protocol IV.

Precipitation of Antibacterial Serum Type "A" by Toxin.*

		.05 cc. of Type "A" antibacterial serum		
		Toxic filtrate of 9 day Type "A" culture (100,000 M.L.D. per cc.)	Formalinized filtrate of 16 day Type "A" culture (originally 1000 M.L.D per cc.)	Formalinized filtrate of 24 day Type "B" culture (control)
Amount of antigen (filtrate)	1.000	−	−	−
	.750	−	−	−
	.500	−	+	−
	.300	−	+	−
	.200	+	++	−
	.150	+	++	−
	.100	++	++	−
	.075	++	+	−
	.050	++	+	−
	.030	++	+	−
	.020	+	+	−
	.015	+	−	−
	.010	+	−	−
	Control of antigen	−	−	−
	" " serum	−	−	−

* This serum contains no antitoxin.

Not every individual organism in a culture of *B. botulinus* is a toxin producer. Single cell cultures produce toxins of various strengths, and occasionally a culture will yield no toxin at all (9). By means of single cell culture (Barber's method), a Type B strain was isolated which failed to produce toxin, but which by morphology, staining reaction, and by agglutination was identified with the mother and with sister strains. When the non-toxic filtrate of this strain was combined with a known antitoxin, flocculation resulted (Protocol V).

Filtrates of 9 day old cultures of this non-toxic strain were used to immunize guinea pigs. Since these filtrates were wholly non-toxic, it was not necessary to formalinize them. After an appropriate succession of subcutaneous injections, the animals were tested and found not to be resistant even to a single lethal dose of Type B toxin, and

Protocol V.

Precipitation of a Type "B" Antitoxin by the Filtrate of an Atoxic Type "B" Variant.

Amount of antigen (atoxic filtrate)	.05 cc. of Type "B" antitoxin	Amount of antigen (atoxic filtrate)	.05 cc. of Type "B" antitoxin
cc.		*cc.*	
.500	+	.050	+
.300	+	.030	−
.200	+	.020	−
.150	++	.015	−
.100	+	Control of antigen	−
.075	+	" " serum	−

Protocol VI.

Precipitation of Antifiltrate Serum (of Type "B" Atoxic Variant) by the Atoxic Filtrate Itself and by a Type "B" Toxin.

Amount of antigen	.05 cc. of antifiltrate serum Type "B"		Amount of antigen	.05 cc. of antifiltrate serum Type "B"	
	Toxic filtrate of Type "B" 24 day culture 100 M.L.D. per cc.	Atoxic filtrate of Type "B" variant 9 day culture		Toxic filtrate of Type "B" 24 day culture 100 M.L.D. per cc.	Atoxic filtrate of Type "B" variant 9 day culture
.500	−	−	.050	−	−
.300	<+	+	.030	−	−
.200	<+	+	.020	−	−
.150	+	++	.015	−	−
.100	<+	+	Control of antigen	−	−
.075	−	+	" " serum	−	−

the serum of the animals failed to protect mice against the homologous toxin. However, the immune serum agglutinated the non-toxic variant and also the bacteria of the toxic mother strain. It flocculated the filtrate of the atoxic variant and at approximately the same point flocculated an old toxin (Protocol VI).

Immunization with Washed Atoxic Variant.

The flocculation of Type B toxin with the serum of animals immunized by the 9 day filtrate of the atoxic variant of this strain was weak and confined to a narrow zone. If the phenomenon is, as our experiments suggested, actually an antibacterial precipitation, it should be possible to increase the precipitating power of the serum by immunizing the animals with the washed culture of the variant.

Accordingly, animals were immunized with the washed bacteria of the atoxic variant, and the serum obtained was found to precipitate the homologous Type B toxin, but more abundantly and in a wider zone (Protocol VII). This serum contained no protective antibodies.

Protocol VII.

Precipitation of Antibacterial Serum (of Type "B" Atoxic Variant) by a Type "B" Toxin.

Amount of antigen, Type "B" toxin 300 M.L.D. per cc.	.05 cc. of Antibacterial Serum "B"	Amount of antigen, Type "B" toxin 300 M.L.D. per cc.	.05 cc. of Antibacterial Serum "B"
.500	−	.050	+
.300	−	.030	+
.200	<+	.020	−
.150	+	.015	−
.100	+	Control of antigen	−
.075	+	" " serum	−

Is the Antitoxin Precipitated?

In specific precipitations, the bulk of the precipitate has repeatedly been shown to be composed mainly of the antibody-carrying globulin. If the precipitate which occurred in the Ramon test took place at the expense of the antitoxin, the latter must have been appreciably diminished in concentration in the supernatant fluid.

We tested quantitatively the antitoxic content of the supernatant fluid of the "indicating tube," at the center of the zone (1), using as antigen a formalinized filtrate, paralleling this with a tube that contained the same amount of antitoxin, but in which a heterologous flocculation was made to take place, since it was considered possible that some of the antitoxin might be carried down by adsorption on the precipitate formed. A third tube contained antitoxin diluted with broth

to the same extent. The quantities of the reagents used were those that in preliminary tests had been determined as optimum.

After the usual incubation a heavy precipitate appeared in Tubes A and C; Tube B remained clear (Protocol VIII). The flocculus was centrifuged thoroughly, and the supernatant fluids were titrated for comparison of their antitoxic value by intraperitoneal injection in mice, each of which received a single M.L.D. of toxin. The results are given in Protocol VIII.

It is evident that there is practically no difference in the protective power of the serum before (B) and after its flocculation with its own

Protocol VIII.

		Tube A	Tube B	Tube C
		3 cc. anatoxin *Botulinus* A 1 " antitoxin " "	3 cc. broth 1 " antitoxin *Botulinus* A	1.5 cc. broth 1.0 " horse serum .5 " anti-horse serum 1.0 " antitoxin *Botulinus* A
		Each tube centrifuged at high speed and supernatant fluid carefully removed, combined with toxin in doses shown, and injected intraperitoneally in mice. Effect on the mice:		
Toxin "A"	Amount of the supernatant fluids			
.00001	.01000	Survived	Survived	Survived
.00001	.00500	"	"	"
.00001	.00250	"	"	"
.00001	00200	"	"	"
.00001	.00150	"	"	"
.00001	.00100	Died in 20 hrs.	Died in 80 hrs.	Died in 40 hrs.
.00001	.00075	" " <36 "	" " 44 "	" " <16 "
.00001	None	" " <16 "	" " <16 "	" " <16 "

antigen (A), except for such small amount of antitoxin as may be carried down with the precipitate by physical means (see Protocol VIII, Tube C). The antitoxin as such appears to take no part in the precipitation phenomenon of Ramon.

Removal of the Precipitable Substance.

It has been shown (17) that acidification of toxic filtrate is followed by precipitation of the protein without reduction of the toxin content. The effort was made to separate the toxic elements of the

filtrate from the protein by this technique in order to see what effect such treatment might have on the precipitation by homologous sera.

To the toxic filtrate of an old culture, normal hydrochloric acid was added until a precipitate appeared. This was removed rapidly by centrifugation and resuspended in normal salt solution. The hydrogen ion concentrations of both the supernatant liquid and of the resuspended precipitate were carefully adjusted to neutral. At this point both solutions were clear. The acid and alkali used diluted somewhat the original toxin, so that a similar quantity of water was

Protocol IX.

Effect of Acid Coagulation on Specific Precipitation of Toxic Filtrates.

			.05 cc. of antitoxic-antibacterial serum		
			Supernatant fluid neutralized after acid coagulation	Acid coagulum redissolved in normal saline and neutralized	Control dilution of the original toxin
Titration by precipitation	Amount of antigen	.50	−	+	+
		.30	−	+	+
		.20	−	+	+
		.15	−	−	+
		.10	−	−	+
		.075	−	−	+
		.050	−	−	−
		.030	−	−	−
		.020	−	−	−
		.015	−	−	−
		.010	−	−	−
	Control of antigen		−	−	−
	" " serum		−	−	−
Titration of toxicity in mice	Amounts given intraperitoneally	.0003	Died in <21 hrs.	Died in <21 hrs.	Died in <21 hrs.
		.0001	" " <21 "	" " <21 "	" " <21 "
		.00003	" " 42 "	" " 40 "	" " <21 "
		.00001	Survived	Survived	Survived

added to a sample of the original toxin to serve as a control. The three mixtures were then titrated in mice and by flocculation (Protocol IX).

The supernatant fluid lost only a small amount of its toxin and this toxin was adsorbed on the precipitate and was demonstrable there. However, the supernatant fluid did not precipitate the antitoxic-antibacterial serum. The acid coagulum, redissolved and readjusted to the neutral point, did produce the specific precipitate with the antitoxic-antibacterial serum.

DISCUSSION.

A method for the titration of diphtheria toxin and antitoxin *in vitro* would constitute so great an improvement over the biologic one, both because of the elimination of many uncertain factors connected with all tests on animals, and from the point of view of economy, that the publications of Ramon immediately attracted wide attention and stimulated investigation of its reliability. Reports of its usefulness because of close agreement with titrations by the Ehrlich biologic technique soon appeared in the literature (10, 2, 3), and the method was adopted as a routine procedure in many laboratories.

Our study of the phenomenon in the case of the toxin of *B. botulinus* has yielded results that show the precipitation to be entirely independent of either the toxin content of the antigen or the antitoxic content of the serum. It is found that atoxic filtrates precipitate antitoxic sera, and purely antibacterial sera are precipitated by active toxins, the width of the zone and the amount of the precipitate depending apparently upon the amount of bacterial protein present in the antigen used to produce the immune serum. The conclusion, therefore, that the precipitation in this instance is not due to union of toxin and antitoxin, but that it is a purely antibacterial precipitation is unescapable.

How then are we to account for the close agreement that so many workers have reported between the *in vitro* and *in vivo* methods of titration in diphtheria? The answer to this question is, we believe, to be found in the fact that the production of diphtheria toxin has been standardized to such an extent that almost all laboratories follow the same technique. The preparation of the medium, its reaction, the age of the cultures before filtration, and other factors are almost identical (11), and even the strain is the well known standard Park and Williams No. 8.

As the result of this uniformity of preparation, the relation between the toxin content and the concentration of bacterial protein in the culture filtrates tends to be constant. Consequently, the concentrations of antitoxin and of antibacterial antibody in the sera produced by immunization of animals with these filtrates bear sufficiently fixed relations to one another to secure comparable results by both methods

of titration. When the composition of the bacterial filtrate changes, the results of titration by precipitation fail to agree with those of the animal test.

We have not undertaken to repeat with diphtheria toxin the tests made with the *botulinus* toxin, but as our experiments progressed, we noted in the literature that discrepancies, giving indirect support to our contention, had actually occurred in the practical use of the Ramon test (12–14). Moreover, Moloney and Weld (15) investigated the neutrality of the indicating tube and reported that a deviation of 300 per cent in either direction from the neutral point is possible; and they observed incidentally that the toxin-antitoxin precipitation in diphtheria bears a relation to the concentration of agglutinins in the serum, and thus confirm our preliminary results (8).

Zingher (16) found that after he had removed a precipitate induced by the formalinization of toxins containing tricresol, the anatoxins gave absolutely no precipitate with corresponding sera, although they remained highly antigenic, a condition which he explains in the light of our findings (8).

The demonstration that the phenomenon of Ramon is a specific antibacterial precipitation explains many discrepancies which have been reported. It indicates also the inapplicability of the Ramon test to the detection of the toxin of *B. botulinus* in the blood of animals fed with the toxin, since it is the toxin itself that is absorbed from the intestinal tract, the accompanying bacterial protein having been digested by the alimentary enzymes.

SUMMARY AND CONCLUSIONS.

1. Animals immunized with the formalinized filtrates of young toxic cultures of *B. botulinus* produce an antitoxic serum poor in precipitins.

2. Animals immunized with the formalinized filtrates of old and partly autolyzed toxic cultures produce an antitoxic serum containing precipitins.

3. Animals immunized with toxin-free autolyzed bacteria produce a serum free from antitoxin but rich in specific precipitins.

4. Animals immunized with the filtrates of an atoxic variant produce a serum free from antitoxin but rich in precipitins for the homologous toxin.

5. Animals immunized with the washed bacteria of the atoxic variant produce a serum that contains no antitoxin, but is rich in precipitins for the homologous toxin.

6. Removal of the precipitins by flocculation with a non-toxic antigen does not materially reduce the antitoxic value of a serum.

7. Removal of the proteins of the antigen by acid coagulation removes the specific precipitable substance.

8. All the sera that contain precipitins produce the specific flocculus when combined with homologous toxins, anatoxins, or with the filtrates of the atoxic variant. The flocculation is restricted within the type. The amount of the precipitate and the width of the zone vary approximately with the estimated amount of bacterial protein in the antigen that is used for the immunization of animals.

We conclude, therefore, that the toxin-antitoxin flocculation is a specific bacterial precipitation phenomenon.

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EXPERIMENTAL GLOMERULONEPHRITIS INDUCED IN RABBITS WITH THE ENDOTOXIC PRINCIPLE OF STREPTOCOCCUS SCARLATINÆ.*

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Plates 19 to 21.

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In the present communication we wish to report the results obtained in an effort to produce toxic effects in rabbits with the culture lysate of certain strains of *Streptococcus scarlatinæ*. The work was undertaken because of our previous failure to induce toxic effects in the rabbit either with cultures of the specific organism or the culture filtrate. Furthermore we were unable to infect the rabbit with large amounts of living cultures of scarlet fever streptococci.

The fact that in human scarlet fever there is so frequently a nephritic complication, presumably toxic in origin, also prompted attempts to induce the nephritis experimentally. We assumed that the streptococcus of scarlet fever *in vitro* would yield a soluble toxin since the results of Dochez[1] lead him to conclude that both the natural immunity in human beings and the experimental immunity are antitoxic in nature.

Three different isolations of the specific hemolytic streptococcus of scarlet fever were employed in our present study of the nature and effects of the toxic principle. Two cultures, one designated "Harrison," the other "Tyler," were supplied us by Dr. Dick of Chicago, while the third culture was one of our own which had been recovered from the blood of a case of human scarlatina.

For the purpose of determining the presence of toxin in culture, culture filtrate, and culture lysate, separate series of rabbits were injected with materials subcutaneously, intradermally and intravenously with varying quantities of each.

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The filtrates were obtained from nutrient broth-grown cultures and from saline suspensions of streptococci cultivated upon blood agar slants. The cultures on solid medium were grown for periods ranging from 48 hours to 1 week at 37°C. before they were emulsified in saline and filtered through N or V Berkefeld filters. The leucocytes and temperatures of the inoculated rabbits were noted daily for reaction, and observations were made of the areas injected intradermally. All animals dying as a result of the injections were autopsied and the tissues were studied both grossly and microscopically. Other animals were sacrificed during the course of the experiments and a careful study was also made of the various tissues.

To determine whether the active principle of *Streptococcus scarlatinæ* (lysate) induces a characteristic intradermal reaction and to compare the cutaneous lesion with that produced by culture filtrate (Dick test), a series of experiments was carried out upon the immune and non-immune human subjects as well as upon the normal and immune rabbit. The filtered streptococcal lysate which was employed was prepared in the belly cavity of the immune rabbit and *in vitro* by treating the saline-washed streptococci with homologous immune serum. The culture filtrate used was obtained from nutrient broth in which the scarlatinal streptococci had been grown at 37°C. for periods ranging from 2 days to 3 weeks.

EXPERIMENTAL.

Experiment 1 (*Infectivity of Scarlatinal Streptococci*).—Nine full grown healthy rabbits were inoculated with 5 mil quantities of 48 hour cultures of *Streptococcus scarlatinæ* (Dick's "Tyler," "Harrison" and one of our own strains). Each animal received the entire surface growth of two 24 hour cultures from blood agar slants which were washed off and suspended in 5 cc. of normal sterile saline. The three culture strains were injected separately in similar amounts into three groups of these animals, through the subcutaneous, intravenous and intraperitoneal routes respectively.

No animal of the series as a result of this first injection developed symptoms of infection though kept under close observation and studied for a period of 2 weeks. The daily temperatures and blood counts remained normal. There was no local reaction at any site of inoculation. Blood cultures that were made from a number of the animals during the 1st week following the injection were uniformly negative.

Two of the animals of this experiment, after an interval of 2 weeks, were again injected with 5 cc. of the same culture as was used for the first injection. No infection ensued nor was there any apparent toxic effect, the animals remaining perfectly well throughout the period of observation.

Two other animals of Experiment 1 received a second injection intraperitoneally of 10 cc. of the originally used cultures (growth of four blood agar slants). The interval between the first and second injections was 21 days. Both animals appeared toxic the day following the inoculation; however, complete recovery occurred 24 hours later. Two other animals of the series were inoculated intraperitoneally with a suspension of streptococci which was the growth from twelve blood agar slants (24 hour cultures). Within 2 hours after the injection the rabbits were seriously ill, dying 6 and 8 hours later respectively. Cultures made from the blood and other organs at autopsy were sterile.

It would seem in the light of these failures to infect, that the rabbit is refractory to large numbers of virulent scarlatinal streptococci, at least for the strains and in the dosage employed. Though not susceptible to infection after repeated injections of virulent scarlatinal streptococci, the rabbit often shows marked toxic effects as a result of a second injection of the homologous culture, especially when the interval between the first and second inoculations is at least 10 days. While the cultures employed by us are not pathogenic for rabbits they seem to be split up by the animal that has been previously sensitized, in consequence of which a toxic principle is liberated. Undoubtedly the streptococci of the first injection succumb in the animal body, and through the action of the derived disintegration product, the immunity mechanism of the rabbit produces a specific lysin. In this way can be explained the toxic symptoms occasioned in the animal as a result of the second injection of culture. On the other hand, the absence of any immediate or later toxic effects upon the animal that has been previously injected with living streptococci, may be accounted for through the presence of insufficient endotoxin extant at any time to induce symptoms.

Experiment 2 (*Culture Filtrate*).—A second lot of animals was inoculated to determine if the culture filtrate of *Streptococcus scarlatinæ* contained a toxic principle. For this purpose eighteen full grown normal rabbits were inoculated with a single dose of scarlatinal culture filtrate. Sets of three animals each received separately 10 mil quantities of the filtrate intravenously, subcutaneously and intraperitoneally. Another set of three animals each was injected with 0.5 to 2.0 mils of culture filtrate intradermally. The filtrate was prepared from cultures which had been grown in 250 cc. of hydrocele broth at 37°C. for 14 days. Only filtrates of cultures showing a heavy homogeneous bacterial cloud were employed. Filtrates were also used from cultures grown on blood agar slants for 2 days at 37°C. All filtrates were obtained by means of the Berkefeld N or V filter, and tested before using for sterility.

It is noteworthy that no animal of the series gave any evidence of toxemia or other reaction following the injections. The intradermal sites of inoculation were negative aside from the non-specific redness noted about the needle point. Animals of this series which were subsequently inoculated with a second dose of the homologous culture filtrate and with 10 cc. of a heavy emulsion of living streptococci showed no ill effects. These experiments proved that large amounts of streptococcal filtrate from cultures grown for 2 weeks in nutrient broth contain no toxic principle for the rabbit. Furthermore the experiments indicate that the scarlet fever cultures employed are not capable of elaborating a soluble toxin.

Experiment 3 (*Immunization*).—A third lot of animals was immunized against living culture of scarlet fever streptococci for the purpose of using them later in the preparation of streptococcal lysate. Seven full grown normal rabbits were injected subcutaneously with saline suspensions of 48 hour growths of streptococci from blood agar slants. The animals received two injections weekly for a period of 1 month. The first dose was one-half of the growth of a blood agar slant which amount was doubled for each succeeding dose. The last dose was approximately the growths of four slants.

Three animals of the series died during the course of immunization but apparently not from infection as cultures prepared from the blood were negative. Death was probably due to intoxication. 1 month after the last injection of antigen (living culture) the rabbits were tested for the Pfeiffer phenomenon and when positive were used later for the *in vivo* preparation of streptococcal lysate. The highly immune animals of this experiment completely split up the living homologous culture that was introduced into the peritoneal cavity and allowed to remain there for 2 or 3 hours. The material recovered from the peritoneal cavity of these animals formed the bacteriolysate used in Experiment 4.

Experiment 4 (*Streptococcal Lysate Production*).—Eight full grown healthy rabbits which had been previously immunized were employed in this test. The living cultures of the homologous organism were introduced by syringe into the peritoneal cavity of the immune animals. As much as 50 mils of culture were introduced which amount was the total 48 hours growth upon eighteen blood agar slants. 2 to 3 hours after the intraperitoneal injection a number of the animals was sacrificed and the peritoneal fluid collected and filtered. Other animals of this series were permitted to live in order to observe whether any ill effects would result to the animal from a longer sojourn of the culture in the peritoneal cavity. Microscopic examination of the peritoneal fluid of the animals sacrificed showed no cocci or microorganisms of any kind, and cultures prepared with 1 mil quantities of the removed peritoneal material remained sterile which proved there had taken place a complete lysis of the introduced microorganisms.

The amount of peritoneal fluid recovered from the sacrificed animals ranged from 15 to 30 cc., and depended to some extent upon the quantity of culture fluid originally introduced. As a rule, approximately two-thirds of the fluid volume introduced was recovered from the belly cavity within 2 hours afterwards. The collected peritoneal material was always cloudy and of a fluid consistence. After filtration, which was immediately carried out, there usually formed in the clear filtrate a veil-like clot.

Three of the immune animals of this series which had received 30 mils of an emulsion of viable streptococci intraperitoneally, and were not sacrificed for collection of lysate, developed after 24 hours, symptoms of toxemia. The animals were sick for several days; however, they eventually recovered, and have since received two or more intraperitoneal injections of 30 mils of viable culture without showing any ill effects.

Another immune rabbit of this series which was allowed to live after the intraperitoneal injection of 30 mils of viable streptococci, suddenly developed severe toxic symptoms 6 hours afterwards and died 2 hours later. Previous to the onset of symptoms the animal appeared perfectly well. Cultures prepared at autopsy were negative. Still another immune animal of this series which was not sacrificed for lysate collection after having received 20 mils of streptococci (bouillon suspension) into the belly cavity, showed the first signs of toxic effect 5 days later. Paralysis of the lower extremities developed the day following and the animal lingered for several days, finally dying. Streptococci were not recovered at autopsy. In connection with the animals of Experiment 4 it is significant that in no instance did infection occur following the introduction of massive doses of streptococci into the peritoneum.

Experiment 5 (*Toxic Effects of Streptococcal Lysate*).—To determine whether the bacteriolysate contained a toxic principle a number of normal half grown and larger rabbits were injected by various routes with different quantities of the filtered lysate. 1 mil quantities (equivalent to the growth of one agar slant) were given intravenously and subcutaneously, and 0.1 mil intradermally. The normal rabbits receiving 1 mil of filtered lysate developed well defined symptoms and signs of toxemia in 8 to 36 hours after the injection. The lethal dose was effective over a period ranging from 6 hours to several weeks. Many of the injected animals exhibiting toxic effects showed a temperature as high as 108°F., leucocytosis of 30,000 and paralysis. In no animal was there noted an exanthem. There was a cutaneous reaction at the site of inoculation in some of the animals that received the lysate intradermally. The autopsy findings in the rabbits that died invariably revealed a swollen and congested condition of the internal organs particularly the kidneys. The protocols of representative animals of this experiment are given below.

Rabbit 1.—(Feb. 22.) Full grown normal animal was injected with 5 cc. of filtered peritoneal lysate which had been previously prepared in the belly cavity of an immune rabbit from Dick's "Harrison" culture. As calculated this dosage

is approximately the product of 1 billion streptococci. The animal first showed symptoms of toxemia 6 hours following the injection, and became paralyzed in the lower extremities 12 hours later. During the illness the temperature ranged from 104.2–108.2°F. The leucocytic count dropped from 15,000 to 8000. The animal died 60 hours after the injection, showing symptoms of uremia.

At autopsy the gross anatomical findings were as follows: Heart, flabby and distended, myocardium pale in color and friable. Liver enlarged, soft and dark red in color. Kidneys swollen, and cortex studded with punctate hemorrhages. Lungs and other organs negative. It is noteworthy that there was no evidence of intercurrent infection.

Rabbit 2.—(Mar. 1.) Received intravenously 10 mils of filtered lysate (Dick's "Harrison" culture) which corresponded to the product of approximately 1 billion organisms. 24 hours after the inoculation the animal became ill. Temperature 105° F. and the leucocytic count 17,000. On the 2nd day after the onset of symptoms paralysis in the hind legs appeared. The fever persisted and the leucocytic count rose to 48,000 the day before death which was 1 month after the inoculation. Cultures from the blood and internal organs were negative.

The autopsy findings were not remarkable except for the kidneys which were swollen and contained greatly enlarged glomeruli. The liver, spleen and heart presented the usual signs of toxemia.

Rabbit 3.—(Apr. 24.) Normal half grown animal was inoculated intravenously with 10 mils of filtered lysate (approximately the product of 1 billion streptococci—Dick's "Tyler" culture). Signs of profound toxemia appeared in 14 hours after the inoculation, at which time the temperature was 107°F. and the leucocytes 22,000. There was no paralysis or exanthem. The animal died 2 days later and at autopsy showed marked gross changes of a toxic nature in the heart, spleen, liver and kidneys. The latter were cloudy and speckled with petechiæ.

Rabbit 4.—(Feb. 26.) Full grown healthy animal which had been previously immunized against *Streptococcus scarlatinæ* (Dick's "Tyler" strain) was injected intraperitoneally with the saline washings of thirty-six blood agar slants of the homologous organism. The interval between the last immunizing dose and the present intraperitoneal injection was approximately 1 month. The animal appeared sick the next day and 2 days later developed paralysis of the lower extremities. Though there was recovery from the acute illness the animal died subsequently (27 days after the intraperitoneal inoculation). At autopsy there were found the usual toxic changes for the internal organs. The gross changes in the kidneys were striking in that they appeared confined to the glomeruli.

Rabbit 5.—(May 26.) Large Angora animal was injected intravenously at 10.30 a.m. with 10 mils of filtered lysate (Dick's "Harrison" strain). No immediate effects from the injection were noted. At 4.30 p.m. (6 hours later) the animal was found dead in the cage but still warm.

Autopsy showed remarkable gross changes only for the kidneys. Both organs were intensely congested, soft and swollen. On section the cut surface presented

a peculiar rose hue suggesting hemolyzed blood. The congestion was distinctly demarkated for the zone intermediate between the cortex and base of the pyramids. The glomeruli were swollen, red and bleeding. Anatomical diagnosis:—acute hemorrhagic glomerulonephritis (fulminating).

Cutaneous Reaction.

To determine whether the lysate of scarlatinal streptococcus induces a skin reaction, and to compare the lesion with that produced by culture filtrate (Dick test) a series of experiments was carried out upon immune and non-immune volunteers as well as upon the normal and immune rabbit. The filtrates of streptococcal lysate employed in the tests were of two kinds; namely, one which had been prepared in the belly cavity of the immune rabbit and the other made *in vitro* by treating the saline-washed streptococci with specific immune serum. The culture filtrates used in the test were also of two kinds; one was obtained from a 2 weeks old bouillon culture, and the other from a broth culture which had been allowed to grow only for 2 days at 37°C.

Experiment 6 (*Human*).—Six human volunteers, including three known non-immunes, were intradermally injected into different areas on the inner aspects of the forearms with 0.2 mil quantities of the filtered streptococcal lysates and culture filtrates respectively. Each subject received simultaneously into separate areas of the skin the various culture filtrates and lysates under consideration. The right arm was used for the intradermal injection of the filtered lysates and the left arm for the culture filtrates. Similar amounts (0.2 mil) of normal sterile saline and bouillon were separately injected into the skin as controls.

The three non-immunes developed in 24 to 36 hours typical erythematous reactions about the injection sites where the two lysates were introduced. The inoculation sites on the corresponding arm which received the two kinds of culture filtrate, showed only a reaction for the "older filtrate." This reaction while definitely positive was not as prompt in appearing nor as intense in character as was the reaction in the other arm which received the streptococcal lysates. The controls were negative; likewise no typical reaction was noted for the culture filtrate prepared from 2 day old cultures.

Experiment 7 (*Rabbits*).—Six rabbits were used, four normals and two immunes, for testing the skin reaction to streptococcal lysate and culture filtrate. In the normal animals of the series no reaction occurred for either the lysate or culture filtrate. We were unable to even induce an intradermal redness with 5 mils of the culture filtrate; however, for the immune animals a marked inflammatory reaction appeared at the site of inoculation following the injection of 1 mil dosage of lysate.

The results of these experiments with the toxic principle of scarlatinal streptococci would seem to show that the skin reaction in non-immune humans which is obtained by intradermal injections of culture filtrate, is after all due to an endotoxin and not to a soluble toxin. A comparison of the reactions shows that the human skin is even more sensitive to intradermal injections of the streptococcal lysate than it is to the bouillon filtrate of the specific culture. While the filtrates from streptococcal cultures which have been grown for 2 weeks in nutrient broth, give rise to the intradermal reaction in non-immunes, the filtrates from 2 to 3 days old bouillon-cultured streptococci fail in our hands to produce a skin reaction. Since the human skin reaction (Dick test) is induced only with the older culture filtrate and equally as well with the streptococcal lysate it may be assumed that the specific exciting agent in each instance is the product of disintegrated streptococcal cells. It would seem that extremely small quantities of the product derived from dissolved streptococci are capable of causing the cutaneous reaction in the human non-immune. Therefore it is reasonable to assume that the products of autolysed dead streptococci which certainly occur in culture are contained in the culture filtrate, and could explain the intradermal reaction following its introduction.

Pathology.

The significant lesion occurs in the kidney of rabbits reacting to the intravenous injection of filtered streptococcal lysate. Apart from the nephritis induced in these animals, the lesions in other organs are those commonly seen for a variety of bacterial poisons; namely, congestion of the smaller vessels, tissue edema and parenchymatous degenerations. The central nervous system was not examined. The degree and character of the experimental streptococcal nephritis depend upon the dosage of the lysate and the length of time the inoculated animal survives.

It is noteworthy that the rabbits injected intraperitoneally and subcutaneously with the lysate often fail to show lesions in the kidney, and when changes do occur in this organ they are comparatively mild and of no special significance. The most striking and significant

lesions in the kidney follow the intravenous administration of the filtered lysate.

The gross appearance of the affected kidneys varied from a barely perceptible cloudy and swollen condition to one in which the organ is much enlarged, capsule tense and the cortical substance studded with punctate hemorrhages. On section the cut surface is mottled dark red in color and the normal markings are obscured. The small hemorrhages in the cortex seem to correspond, for the most part, to the location of the glomeruli. Other tufts appear swollen, dark red and elevated. The capsule is not adherent to the cortical substance, nor is there any gross evidence of interstitial change.

The microscopic study of the kidney sections fixed in formalin and Zenker's fluid and stained with hematoxylin and eosin, reveals an acute glomerulonephritis as the outstanding lesion. Occasionally the corresponding tubule is involved though often only the convoluted portion. The glomerular change ranges from a simple acute hyperemia of the tuft capillaries to a marked congestion and serum extravasation and hemorrhage into the capsular spaces. In these instances the capillary whorls appear to be pushed to one side or partly crowded out of Bowman's capsule.

The sections of kidney from rabbits that survive the immediate effects of the lysate, though not completely recovering, show remarkable changes in the Malpighian bodies in the form of hyaline thrombi of the tuft capillaries. Microscopic fields in which there are six to ten glomeruli, show hyaline masses in the vessels of fully one-third of these. The convoluted part of the corresponding tubule often reveals an advanced stage of retrograde metamorphosis for the lining epithelium. No change is noted for the tubules not connected with thrombosed tufts. The epithelium appears structurally normal for tubules in which the glomerular portions present lesions of a mild character.

Still another form of glomerular change is noted for the kidney of paralyzed rabbits which died 3 weeks or more after the intravenous injection of streptococcal lysate. The Malpighian bodies contain a marked increase in the number of mononucleated cells lining the tuft capillaries. The picture is not that of an endarteritis as the cells seem to be free in the lumen of the vessels. These cells are not in

evidence for tufts in which shrinkage and complete disintegration have occurred, Bowman's capsule then containing only necrotic remains of vessels and their endothelium. For some glomeruli the necrotic tuft appears shrunken, in shadowy outline and eccentrically displaced by either collections of serum, blood clot or "crescentic" masses of proliferated capsular epithelium. The latter are the so called "epithelial crescents" which invariably begin in the tubule portion of Bowman's capsule and in consequence cause a blocking of the entrance to the corresponding tubule.

Tubular changes are not an early feature of the acute parenchymatous nephritis. Often no epithelial alterations are observed except where the corresponding glomerular tuft is markedly altered. In these instances the tubules show a swollen and granular condition of the lining epithelium especially of the convoluted portion. At this stage desquamation of the epithelium and its appearance in the form of casts are frequently noted.

In none of the experimental rabbits dying in a week to 10 days as a result of the acute nephritis did there occur lesions outside of the parenchyme. In other words, there was no lymphocytic infiltration of the interstitial tissues or proliferative activity of the connective tissue stroma. However, it should be mentioned in this connection that in certain of our experiments where one of the rabbits previously immunized against the streptococcus of scarlatina died as a result of a subsequent injection of the homologous "lysate," chronic interstitial changes were noted in the kidneys. These chronic changes were essentially reparatory and undoubtedly the sequence of some previous injury to the parenchyme. Whether the primary glomerular injury induced experimentally with the streptococcus may inaugurate secondary chronic changes in the stromal tissues can only be conjectured at this writing. Further experiments in which the chronic lesions occur regularly under specific conditions are necessary before any conclusions can be drawn regarding the specificity of the interstitial change or its relationship to the acute glomerular nephritis experimentally produced with *Streptococcus scarlatinæ*.

DISCUSSION.

The experiments which we have carried out upon rabbits indicate that the active toxic principle of the H. *Streptococcus scarlatinæ* is bound up in the cytoplasm of the microorganismal cell. The experiments also show that the toxin of certain strains of scarlet fever streptococci, including the Dick "Harrison" and "Tyler" isolations, is not a secretory product of the living organism. In consequence of this the active principle is in no way comparable to a soluble toxin.

Primary large doses of the scarlatinal streptococci or the culture filtrate injected into the rabbit do not produce a toxic reaction. After several injections have been given for immunization purposes a subsequent unduly large inoculation of the organism may produce toxic effects. In other words, a dose which given primarily would have no effect upon the rabbit will, on the other hand, produce toxic symptoms in the animal that has been partially immunized. This would seem attributable to the liberation of an intracellular or endotoxin from the injected organism by the specific bacteriolysin previously produced in the animal through the action of specific antigen. It cannot be attributed to any free toxin injected, as the same dose or a larger amount is inert when given as a first inoculation.

After immunization of the rabbit the intraperitoneal injection of a large amount of scarlatinal streptococci will produce toxic symptoms and death in from 2 to 24 hours whereas the intraperitoneal injection of a similar quantity into the rabbit which has not been immunized, has no appreciable effect. Manifestly the endotoxin has here again been liberated by the specific bacteriolysin present in the immunized rabbit and the action of this liberated toxin may prove fatal. In procuring our bacteriolysate from the peritoneal cavity of the immune animal which had been given a large dose of the streptococcal culture into this cavity, it was considered preferable to obtain this toxic material in from 1 to 2 hours following the intraperitoneal injection, in consequence, the animal was sacrificed at the end of this period. Through this procedure the greatest amount of the introduced culture material could be recovered and at a time when its endotoxic content demonstrated considerable potency.

While a certain amount of toxin must be liberated following each

immunization dose it is insufficient to injure the animal. It seems also apparent that but little antitoxin is formed from this small amount of liberated toxin since a subsequent intraperitoneal injection will prove fatal, indicating a failure of neutralization of the toxin. That there occurs marked lysis of the organism injected intraperitoneally is demonstrated by the absence of these organisms in smear preparations of the fluid and negative cultural results.

The bacteriolysate *per se* and the filtrate contain a toxic principle as is proven by the fatal effect upon the rabbit in from 6 to 12 hours. This toxin while violent in its action requires large doses to produce such a result and is, therefore, not comparable in potency to the soluble toxin of microorganisms such as *B. diphtheriæ*.

Since *Streptococcus scarlatinæ* is not pathogenic for the rabbit, it is necessary to employ large doses of the toxin to produce a fatal result. However, this same bacteriolysate is more toxic for the human species as is demonstrated by intradermal injections. Cutaneous injection of 0.2 cc. into the rabbit produces no effect whereas this amount when similarly administered to non-immune human subjects produces a marked reaction. Careful standardization of the toxic bacteriolysate has not yet been undertaken. In our experiments any dose less than 5 cc. has not caused death.

It is questionable whether the skin reaction to intradermal injection of specific culture filtrate in humans who are non-immune to scarlet fever, is produced by a streptococcal exotoxin. There is no experimental evidence in the rabbit, at least, to show that the scarlet fever streptococcus elaborates what we are pleased to call a soluble toxin. Certainly for this animal an active toxic principle is not demonstrable in the filtrate of cultures grown in nutrient broth for periods of 10 days to 2 weeks. The endotoxin of scarlet fever streptococci does not preclude its accounting for the exanthem in the human case or the skin reaction in the human non-immune. If we accept the view that scarlet fever is a localized streptococcal infection, we must recognize the fact that the organisms are constantly dying and being destroyed by the host. In consequence, the liberated endotoxin may reach the cutaneous tissues *via* the circulation in sufficient concentration to give rise to the exanthem. An intracellular poison of a pathogenic microorganism is not different from that of a soluble toxin in its specific action upon the tissues.

Since in human scarlet fever there is commonly an acute nephritis, the production of kidney lesions in the experimental animal with scarlet fever streptococci is of significance. The experimental nephritis herein reported for rabbits supports the view that the streptococcus is responsible for the acute toxic nephritis in scarlatina but not necessarily is it inferred that the streptococcus plays a solitary rôle in the production of the scarlet fever symptom complex.

A study of the histopathology of the rabbits dying as a result of the intravenous injection of streptococcal lysate shows pronounced lesions in the glomeruli of the kidneys. The constant occurrence of the lesion in the kidney tuft suggests a selective action of the streptococcal endotoxin and permits of the deduction that the mortality in the experimental animal is the result of an acute hemorrhagic glomerulonephritis. Furthermore, the location and character of the nephritic lesion of the rabbit indicate that the acute nephritis in human scarlet fever is caused by certain hemolytic streptococci.

Since the character of the kidney lesion in the experimental rabbit is in many respects like that of the acute scarlatinal nephritis in man, it constitutes evidence of the possible specific relationship of the streptococcus; however, it is no proof that the hemolytic streptococcus is the only cause of the disease.

SUMMARY.

1. Broth-grown cultures, cultures from blood agar slants and culture filtrates (Berkefeld N or V) of H. *Streptococcus scarlatinæ* are without appreciable effect upon the rabbit, no matter how large the dose or by what route introduced.

2. The active toxic principle of H. *Streptococcus scarlatinæ* for rabbits is intimately associated with the protein of the bacterial cell, and is not given off in the artificial medium during the growth activity of the organism, indicating, therefore, its endotoxic character.

3. The endotoxin is readily obtained from the viable scarlatinal cultures through the medium of the peritoneal cavity of the rabbit immunized against the homologous strain (Pfeiffer phenomenon). The toxic substance thus obtained we have termed a lysate.

4. The rabbit is highly susceptible to the *in vivo* prepared lysate of *Streptococcus scarlatinæ*, at least from the cultures we have em-

ployed. The degree of the toxic effect upon the rabbit depends upon the size of the dose and the route through which it is introduced. The specific effects range from mild to severe and fatal forms of toxemia as indicated by high fever, leucocytosis, paralysis and acute hemorrhagic glomerular nephritis.

5. The experimentally induced nephritic lesions are analogous in kind and variety to those of acute scarlatinal nephritis in man, including the "epithelial crescent" formation, hyaline thrombi of glomerular capillaries, hemorrhage into capsular space and necrosis of capillary tufts.

EXPLANATION OF PLATES.

PLATE 19.

FIG. 1. Section of rabbit kidney showing swollen and distorted glomerulus. Note the hemorrhage in Bowman's capsule which has displaced the capillary tuft and extends into the corresponding tubule.

FIG. 2. Section of rabbit kidney showing early vascular changes in the glomerular vessels. Note the marked dilatation of the tuft capillaries which are distended with red blood cells that have lost their hemoglobin and appear as sharply outlined colorless circles crowding the vessel lumina. The adjacent convoluted tubules are swollen and the lining epithelium granular.

FIG. 3. Section of rabbit kidney showing atrophied tuft and Bowman's capsule more than half filled with proliferated epithelium ("crescent").

PLATE 20.

FIGS. 4 and 5. Section of rabbit kidney showing various proliferative and degenerative lesions in the glomeruli. Note the marked increase in the endothelium of the tufts and the distention and vacuolization of the latter.

PLATE 21.

FIG. 6. Section of rabbit kidney in which the glomerulus is degenerated and pushed toward the vascular end of Bowman's capsule by extensive hemorrhage.

FIG. 7. Shows hyaline thrombi in glomerular vessels. The thrombi are apparently situated in the efferent loops of the capillary tuft.

FIG. 8. Section of rabbit kidney showing exudative and proliferative changes in the glomerulus. The capillary tuft is atrophied and Bowman's capsule is partly filled with extravasated serum. Note especially that in one part of the capsule there is the early formation of the "epithelial crescent."

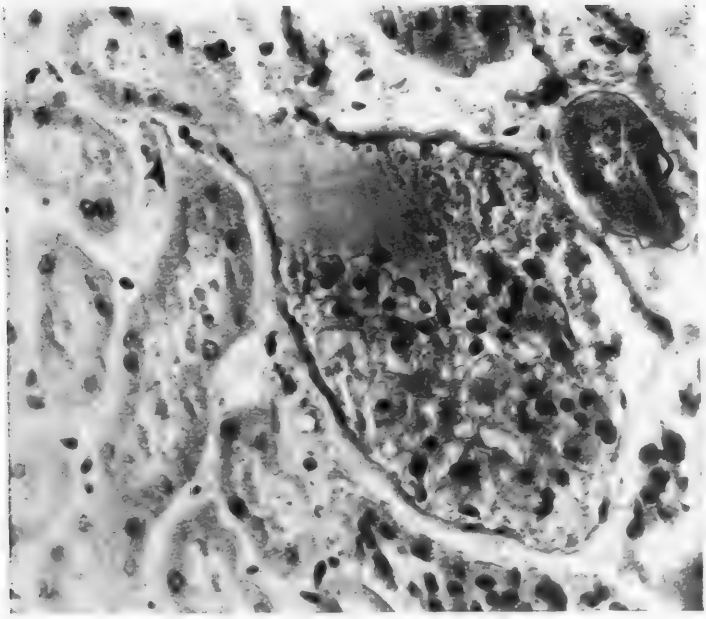

FIG. 1.

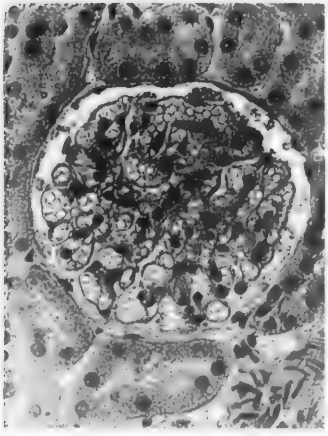

FIG. 2.

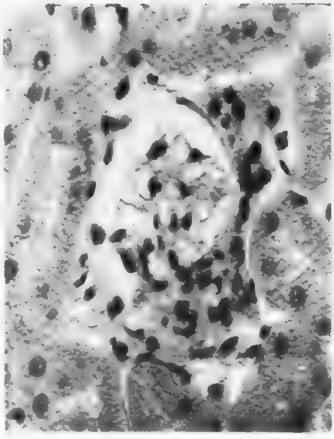

FIG. 3.

(Duval and Hibbard: Endotoxin from *Streptococcus scarlatinæ*.)

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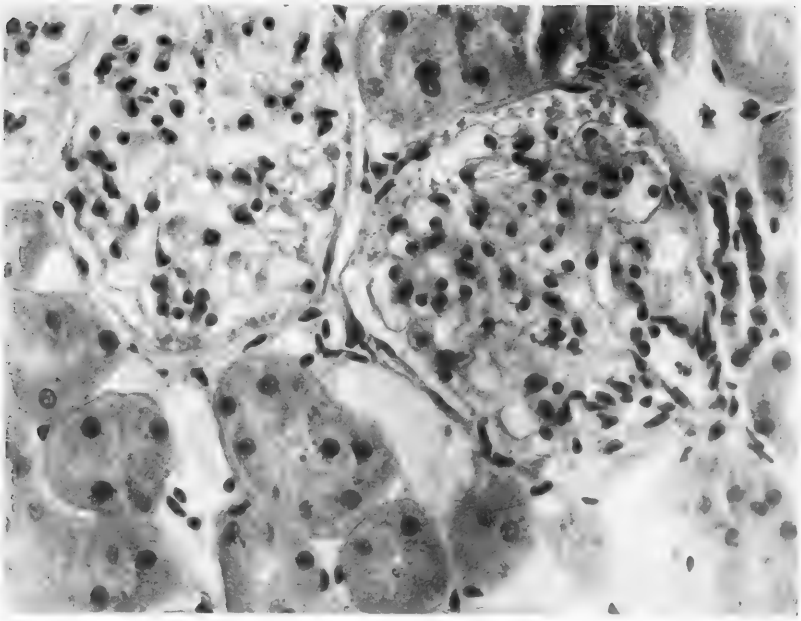

FIG. 4.

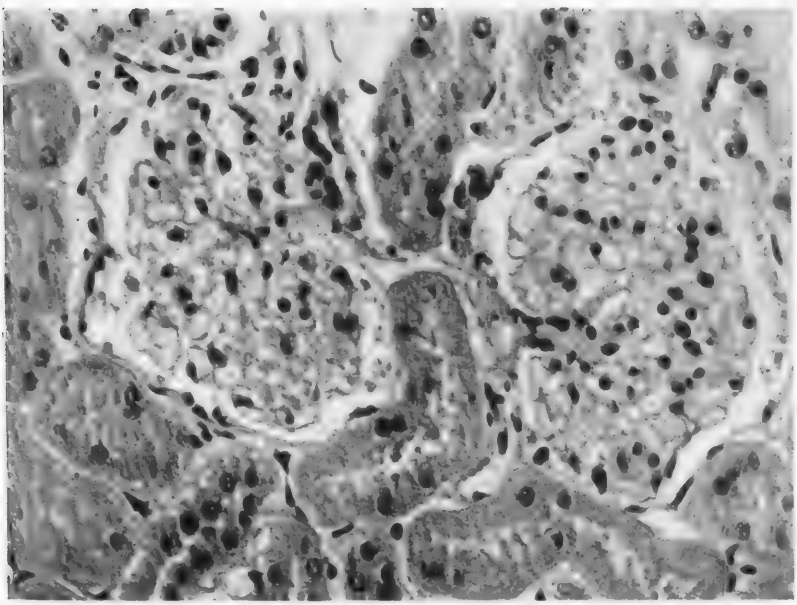

FIG. 5.

(Duval and Hibbard: Endotoxin from *Streptococcus scarlatinæ.*)

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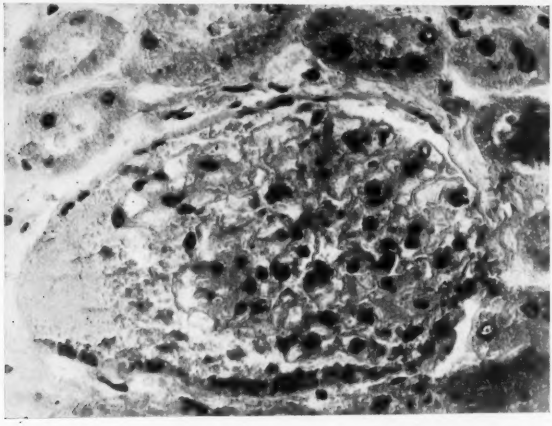

FIG. 6.

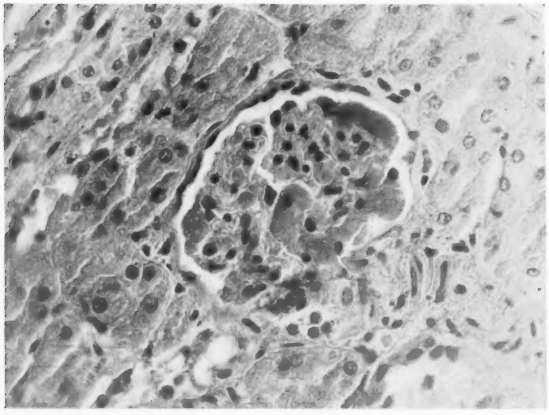

FIG. 7.

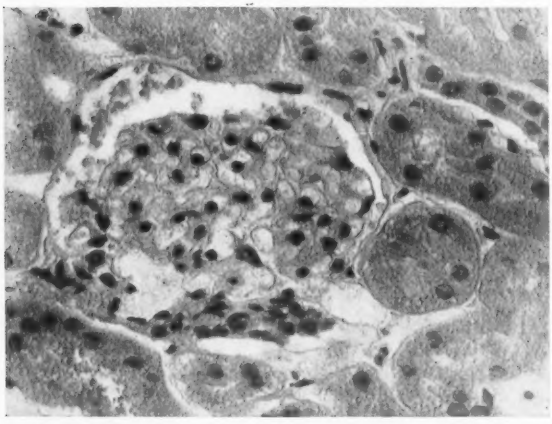

FIG. 8.

(Duval and Hibbard: Endotoxin from *Streptococcus scarlatinæ*.)

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